BREEDING FOR TOLERANCE OF COWPEA TO LOW PHOSPHORUS SOIL CONDITIONS THROUGH PHYSIOLOGICAL AND GENETIC STUDIES

A Dissertation

by

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ABSTRACT

Cowpea (*Vigna unguiculata* (L.) Walp.) is a major food legume across Sub-Saharan West Africa where its leaves, pods and seeds are consumed as food and its residues are fed to livestock as protein rich fodder. However, soils of West Africa are poor in phosphorus (P), a soil macronutrient all crops need for growth. Fertilizer with P is not readily available and is too expensive for West African farmers. This research was therefore, undertaken to identify cowpea lines that inherently grow well in P-deficient soils and use them to breed improved cowpea varieties that require less phosphorus fertilization. A hydroponic phenotypic screening method with silica sand was used to identify cowpea varieties that have tolerance to low soil P as measured by shoot dry biomass production. Both tolerant and susceptible varieties from the screen were further analyzed for root biomass, internal shoot P content, and internal root P content. Seed P, particularly the effect of cotyledon P, and total root production were investigated as physiological sources of tolerance. Tolerant cowpea varieties were crossed with susceptible varieties, and the resulting F_1, F_2 and BC_1 seeds were screened to determine the inheritance and genetic control of tolerance. A Recombinant Inbred Line (RIL) population of a tolerant by susceptible cross was mapped using SSR markers to identify linkage groups or QTL for tolerance to low soil P.

Phenotypic screening results identified four cowpea varieties to have P-deficiency tolerance (Big John, IT97K-1069-6, IT98K-476-8, and TX2028-1-3-1) and three cowpea varieties (Big John, CB-46, and Golden Eye Cream) to have partial P-

deficiency tolerance via high seed P content. All varieties experienced increases in root production under low P treatments relative to normal P treatments. Phenotyping of F_1, F_2, and BC_1 populations showed that low P tolerance is a heritable trait in cowpea with significant additive effects and narrow-sense heritability. Estimates of gene number suggested the tolerance to be a single-gene trait. Mapping linkage groups or QTL for low P tolerance identified QTL in which three SSR markers – CLM0269, 221/222, and CLM0298 – were significantly associated with tolerance and are potential candidates for marker-assisted selection (MAS).

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1. MAIN INTRODUCTION AND LITERATURE REVIEW

1.1. Main Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is a staple food crop for parts of the world, including Brazil, India and much of Africa. In these countries cowpea is often grown for home consumption and not sold for the market. More than 65% of cowpea worldwide is produced in Africa, with Nigeria and Niger producing more than 50% of cowpea worldwide (Gepts and Kuhn 2008). The U.S. is the only developed country with significant cowpea production, and cowpea is more commonly grown and marketed as "black-eyed pea." Cowpea is significant in regions worldwide as an intercrop that replenishes soil fertility, particularly by increasing nitrogen availability. Cowpea is also a renowned heat and drought tolerant crop. For nutrition, cowpea is principally consumed for its seeds that are high in protein, roughly 25%, and vitamins. Cowpea leaves and pods are also consumed in parts of the world and are a source of nutrition for both humans and animals. The yield of cowpea in developing countries is typically 250–400 kg/ha (or 223-357 lb/acre), whereas in the U.S. cowpea yield is typically 2000 kg/ha (or 1784 lb/acre) (Gepts and Kuhn 2008). This stark difference in yield between the two regions is due to drought, insects, disease, and poor soil fertility. This dissertation will address the latter of these problems by breeding for production in low phosphorus (P) soil conditions.

Phosphorus is one of the critical minerals required to sustain agricultural production. Most of the world's current phosphorus supply is from rock phosphate (RP)

which is a bound-form of phosphorus. Rock P is unevenly distributed throughout the world, with Morocco, China, South Africa, Jordan and the U.S. containing a majority of the world's supply, which will likely make RP import and export an issue in future international politics (Schröder *et al*. 2010). Several predictions indicate that current commercial reserves of RP will be depleted within the next 50-100 years (Cordell *et al*. 2009; Schröder *et al*. 2010). Morocco and the west Sahara in Africa contain more than a third of the world's usable RP, and yet much of this RP is being exported to other countries. For these African countries in development, the cost of transporting and manufacturing fertilizer from this RP for their own use is too high. Thus, 75% of African soils are still P-deficient (Cordell *et al*. 2009). Since cowpea is a significant staple crop in African soils that have widespread soil-P deficiency problems, breeding for cowpea's tolerance to such conditions is of importance. Such breeding efforts have already begun, and cowpea lines with tolerance to low P soils have recently been identified (Saidou *et al*. 2011).

These lines display one of two different types of tolerance. The first is P use efficiency (PUE), in which cowpea is able to efficiently use and recycle its seed P supply with minimal uptake of soil P. The second is P acquisition efficiency (PAE), in which cowpea is able to acquire P from soil RP when there is minimal freely available inorganic P (Pi). However the physiological mechanisms responsible for these tolerance traits in cowpea are unknown. Also the genetic basis for this tolerance is unknown. Without knowledge of either the physiological mechanisms or the genetic basis, the

development of cowpea lines for tolerance to low soil P conditions beyond simple selection is difficult.

The *purpose of this research* is to further elucidate the genetic control and physiological mechanisms responsible for tolerance to low P soils and identify the quantitative trait loci (QTL) regulating these traits so that cowpea lines with tolerance to soil P-deficiency can be bred. Tolerant cowpea lines will be identified via phenotyping with a hydroponic silica sand screening method. Data from this screen will be utilized to develop F_1, F_2, BC_1, and recombinant inbred lines (RILs) of 'high x low' crosses of cowpea for tolerance to low P soils. The F_1, F_2, and BC_1, seed will also be phenotyped for tolerance via the hydroponic silica sand screening method to determine the heritability and genetic control of tolerance to low P soils in cowpea. The RILs will be phenotyped via the same method and used to map linkage groups or QTL for tolerance to low P soils in cowpea. The central hypothesis of this research is that *tolerance to low P soils is a heritable trait in cowpea. A high efficiency utilization of seed P and enhanced root growth are responsible for low P tolerance in some cowpea lines. F_1, F_2, BC_1, and RIL seed developed for 'high x low' crosses of cowpea lines for the tolerance to low P soils will screen positively for tolerance and provide the start material for QTL identification of each trait in cowpea using simple sequence repeat (SSR) markers.* The refined knowledge of the physiological and genetic mechanisms for tolerance to low P soils gained from this research will make more accurate and efficient selection and breeding of cowpea lines for soil P-deficiency tolerance possible.

The following objectives will be used to test this hypothesis:

Objective 1. Develop an accurate phenotyping method for tolerance to low P conditions in cowpea lines, and determine associated physiological-level causes. A hydroponic silica sand system was tested as a means to phenotype cowpea lines for low P tolerance with minimized stress effects. Pots with silica sand were watered with nutrient solutions of different P treatments. Plants harvested were analyzed for total shoot dry biomass produced to determine tolerance. Also, root dry biomass, shoot internal P content, root internal P content, and original seed P were analyzed to determine possible physiological mechanisms for tolerance.

Objective 2. Determine the heritability and genetic control of tolerance to low P soils in cowpea. F_1, F_2, and BC_1 seed of 'high x low' crosses of cowpea lines for tolerance to low P soils were phenotyped using a hydroponic silica sand system. Total shoot biomass and root biomass produced were analyzed to determine the heritability and genetic control of tolerance to low P soils in cowpea.

Objective 3. Define QTL regulating tolerance to low P soils in cowpea. SSR markers already identified in cowpea were used to map tolerance to low P soils.

This research will pave the way for the development of P efficient cowpea lines that will overcome low soil fertility problems in Sub-Saharan West Africa. This research will also contribute to understanding physiological mechanisms and the genetic control of tolerance to low P soils. Such knowledge will become more valuable as future supplies of global P become depleted.

1.2. Literature Review

1.2.1. *Cowpea as an agricultural crop*

Cowpea, commonly known as "black-eyed peas" or "pink-eyed/purple hull southern peas" in the U.S., is grown throughout Africa, Southeast Asia, South America, and the U.S. Within the U.S., its production at over approximately 60,000 to 80,000 acres is mostly in California, Texas and the southeastern U.S. (Quinn and Myers 2002). Outside the U.S., its production is largely in Africa, particularly Nigeria and Niger, but other countries with substantial production include Brazil, India, Haiti, Myanmar, Sri Lanka, Australia, and Bosnia-Herzegovina (Quinn and Myers 2002). Cowpea's popularity in Africa is attributable to the edibility and marketability of its many parts -- the leaves, immature pods, fresh seeds and dry grain (Cisse and Hall 2003). Also cowpea is popular because of its fast maturation and short life-cycle. The crop provides food and income during the "hungry period", from roughly August to September at the end of the wet season, when other food is not readily available for parts of Sub-Saharan Africa (Cisse and Hall 2003). Also in parts of Africa, the fodder, or haulms, for cowpea are used for livestock feed.

Cowpea is a favorable agronomic crop for many reasons. It has relatively high drought, heat and shade tolerance. It can grow in many marginal soils, such as sandy soils with variable pH, and fix nitrogen therein. For these traits, cowpea is ideal for intercropping systems. As a food, cowpea is favorable for its high, roughly 25%, protein content and vitamins (folic acid and vitamin B). The protein amino acids in cowpea are complimentary to those found in cereals, and thus cowpea serves as a great nutrition

compliment to cereals. Cowpea yields within the U.S. are typically 1008 to 1512 kg/ha, but yields upwards of 3024 kg/ha have been reported in California (Quinn and Myers 2002). These yields are substantially higher than those in developing countries at around 250-400 (Gepts and Kuhn 2008). On the U.S. market, cowpea is estimated at $0.46/lb dry, and any fluctuations in price are typically due to production and demand factors (FAOSTAT 2009).

1.2.2. *Soil phosphorus availability worldwide*

Phosphorus (P) is a vital yet limited natural resource for agricultural production worldwide. As a natural resource, P is renewable, but natural reserves are being depleted faster than can be restored. P is commonly found as rock phosphate (RP), and this RP has over the last century become the main source of fertilizer production over manure, which was previously the main source (Schröder *et al*. 2010). However, remaining reserves of RP are principally held by a select few countries: Morocco, China, South Africa, Jordan and the U.S. Thus RP has become a subject for international politics, and its exportation and importation worldwide cannot be readily guaranteed.

The depletion of P for agriculture has started to gradually come to the forefront of concern for agricultural scientists, and more predictions of nearing depletion of P reserves have been made. Schröder *et al*. (2010) wrote a compilation of predictions for the estimated reserves of P to be available worldwide: Steen (1998) – 60 to 130 years, Smil (2000) – 80 years, Smit *et al*. (2009) – 69-100 years, Vaccari (2009) – 90 years, and Fixen (2009) – 93 years. All of these predictions were made based on either current extraction rates or predicted slight increases in extraction rate. Predictions for the peak

production of P are also being made based on the amount of RP reserves worldwide. Based on USGS data, Cordell *et al*. (2009) predicts that maximum production will peak at 2035. Given these predictions, recommendations for the conservation of future P resources are gradually being spread. One such recommendation involves the better collection and distribution of human or animal excretions, in a sanitary matter, for use as fertilizer in agriculture (EcoSanRes 2008; Schröder *et al*. 2010). This redirection of wastes is one step for helping solve the problem, but at the same time proper and efficient utilization of RP resources also needs to be addressed.

As serious as the predictions may be, a microcosm for the problem of P unavailability already exists in Africa. Though the world's largest reserves of RP exist in Morocco and the Western Sahara, large amounts of this RP are being exported. The costs of transportation and processing RP into fertilizer have largely made RP an unavailable resource to much of Africa. Instead regions of Africa have had to struggle through sustaining agriculture in soil P deficiency conditions.

As more agricultural and international development research projects are developed, overcoming low soil fertility is a key issue for consideration. As an intermediate step, research for the development of crops to grow well in P deficiency conditions is of importance.

1.2.3. *Screening cowpea for tolerance to low P soils and identifying the physiological mechanisms responsible*

Previous screens have been done to identify cowpea lines that grow efficiently in low soil P conditions and to identify the responsible physiological mechanisms.

Vesterager *et al*. (2006) studied the effects of soil P deficiency on pigeonpea but included cowpea as a control. Vesterager noted that cowpea had increased root hair growth relative to pigeonpea, perhaps as a mechanism to acquire more soil P early since cowpea is one of the fastest growing legumes. However cowpea's P uptake for the volume of soil explored by these extra root hairs was not comparable to the rate of pigeonpea's P uptake for its volume of soil explored. Other studies have also tried to determine correlations between cowpea's root growth and P uptake. A study by Sanginga *et al*. (2000) showed variation in P uptake efficiency by different cowpea lines and suggested root growth differences to be a potential cause. Another study by Krasilnikoff *et al*. (2003) showed there was variation among cowpea lines in P uptake and root growth under low P stress conditions. However it was not clear that this root growth was correlated to P uptake. For example the line Dan Ila showed the most root hair growth but low P uptake. The study suggested that root growth is perhaps for drought tolerance instead.

Pypers *et al*. (2006) studied the exudation of organic acids from cowpea lines in low P soils. They found exudation amounts increased in low P soils, but P uptake was still low because the concentration of P in soil solution was still low. Nwoke *et al*. (2008) also measured organic acid production by soybean and cowpea in low P soils. They only detected citric acid which then had a low correlation to increased P uptake by cowpea. Thus they suggested root length may be more important. Alkama *et al*. (2009) tested cowpea's P-use efficiency in low P soils through the proton efflux of nodulated roots. They concluded there was some correlation between this proton efflux and nodule

specific respiration to cowpea's PUE in low P soils. A study by Jemo *et al*. (2006) identified two cowpea lines that did well in low P soils and experienced increased N_2 fixation. One line was suspected to do well because of better root infection by arbuscular mycorrhizal fungi (AMF), and the other line was suspected to do well because of better root morphology and physiology. Saidou *et al*. (2007, 2011) studied many cowpea genotypes for response to low P, RP, and single sugar phosphate (SSP) in both field and greenhouse environments. They identified low-P tolerance in several lines from field and pot screening. They also noted that increased phosphorus P availability led to decreased AMF colonization and an increased shoot:root ratio.

An unsuspecting factor affecting cowpea's response to low P soil is seed available P. A study in *Phaseolus vulgaris* L. (common bean) and its response to low P soils showed that large-seeded varieties did better than small-seeded varieties (Yan *et al*. 1995). A previous paper they referenced described the importance of taking into account seed P when determining a plant's P uptake from soil (Brookes 1982). In cowpea itself, a genetic evaluation of cowpea's phosphorus utilization showed that larger seeds experienced higher P uptake from soil (Ojo *et al*. 2007). Also when a large-seeded P-uptake efficient line was crossed with a small-seeded P-uptake inefficient line, the backcrosses to each parent produced even larger seeds with more seed P. These backcross lines were even more efficient at P uptake than either parents, the F_1's or the F_2's. A study by Teixeira *et al*. (1999) on bean cultivars showed that plants with a higher seed P were less dependent on soil P supply, and also experienced increased nodulation and nitrogen fixation. A study on soybean by Tang *et al*. (2007) showed that increased

seed P provided early vigor for the plant and led to more nodulation and increased plant efficiency.

1.2.4. *QTL mapping in cowpea*

Several studies have already been done to identify cowpea lines and their physiological traits for tolerance to low P soil conditions. However studies have yet to identify the QTL and genes involved in this tolerance.

Molecular markers are fundamental to identifying QTL and genes responsible for agronomic traits. Molecular markers in cowpea have already been used for genetic diversity studies and the development of genetic linkage maps. Different markers that have been used to study genetic diversity in cowpea are allozymes (Pasquet 1999; Pasquet 2000), microsatellites or simple sequence repeats (SSRs) (Li *et al*. 2001; Diouf and Hilu 2005; Ogunkanmi *et al*. 2008; Asare *et al*. 2010), AFLPs (Seehalak *et al*. 2006; Fang *et al*. 2007; Polegri and Negri 2010), and RAPDs (Ba *et al*. 2004; Diouf and Hilu 2005).

Molecular markers have also been used to develop genetic linkage maps in cowpea. Ubi *et al*. (2000) developed a map from RAPD markers and located QTL for various traits such as days to flowering, pod length, leaf area, etc. Ouédraogo *et al*. (2002) developed a map from AFLP, RFLP, RAPD and biochemical markers. Markers for various traits were placed on the map such as resistance to cowpea mosaic virus (CPMV), *Fusarium* wilt, root-knot nematodes, etc. Muchero *et al*. (2009a) developed a genetic linkage map of cowpea but from EST-derived SNPs. Andargie *et al*. (2011) developed a genetic linkage map from SSRs and identified QTL for seed size and pod

shattering in their map. Sequencing of the whole cowpea genome is underway which will greatly enhance the molecular breeding of cowpea (Timko *et al*. 2008).

As these markers and genetic maps are started, the possibility to identify QTL and the genes responsible for traits in cowpea increases. Recently, Gupta and Goalakrishna (2010) developed unigene-derived SSR markers for cowpea that could be used for mapping. Mapping QTL in cowpea has already been started for several traits – thrips resistance (Omo-Ikerodah *et al*. 2008; Muchero *et al*. 2010a), drought resistance (Muchero *et al*. 2009b and 2010b), bacterial blight resistance (Agbicodo *et al*. 2010), and Macrophomina phaseolina resistance (Muchero *et al*. 2011).

2. ACCURATE PHENOTYPING AND IDENTIFICATION OF PHYSIOLOGICAL CAUSES FOR COWPEA TOLERANCE TO P-DEFICIENT SOILS

2.1. Introduction

Cowpea is the staple legume for Sub-Saharan West Africa where both people and livestock consume cowpea seeds, pods, and leaves. The average cowpea yield in West Africa is 240 kg/ha but estimates exist that yield could be around 10 times higher according to trial testing (Quin 1997, Chimphango *et al*. 2008). This lower yield results from drought, poor soil fertility, disease, and insects. This study addresses poor soil phosphorus (P) levels as one of the causes of lower yield.

Phosphorus is one of the three macronutrients commonly found in fertilizer and is required by plants for normal growth and seed production. While most of the world's soils either inherently or from fertilizer have normal to high P content, the soils in West Africa are deficient in P. This imbalance is due to the fact the African soils are old and weathered and due to high importation and production costs for fertilizer in West Africa. There also exists growing concern that depletion of P will become a worldwide problem. Several predictions have been made that P reserves will be largely gone within the next century (Schröder *et al*. 2010). Approximately 80% of current P reserves are in Morocco, China, Kazakhistan, South Africa, and the United States (Sunkar Resources 2009). When P reserves start to be depleted, P trade could become subject to international disputes.

Research on crops that can efficiently grow with limited P and recycle P back into the system is of high importance for the future security of agriculture. Cowpea is an excellent candidate crop for this research because the majority of cowpea is already grown in P-deficient soils of West Africa. Cowpea is a relatively easy crop to work with because of a short life-cycle (60-day varieties exist) and because cowpea has a diploid genome of just 620 Mbp.

Within current literature, both P-use efficiency (PUE) and P-acquisition efficiency (PAE) have been discussed as favorable traits for increasing crop yield in P-deficient soils. PUE can be defined as "the amount of total biomass, or yield, produced per unit of P taken up" (Hammond *et al*. 2009 and Veneklaas *et al*. 2012). PAE can be defined as "the ability of plants to solubilize and absorb P from the soil" (Moll *et al*. 1982 and de Sousa *et al*. 2012). Mechanisms for PUE in plants can be linked to several traits: better use of stored P within or in translocation between plant tissues, low P demand on the cellular level, and an efficient recycling of P back into the seed for the next generation (Marschner 1995). Mechanisms for PAE can be linked to several traits as well: root architecture, root hairs, mycorrhizal fungi associations, root surface anion exchange capacity, and root organic acid exudation (Marschner 1995). For future production of crops in P-deficient soils, both PUE and PAE traits packaged into crop varieties will be of interest.

This study will focus on P use and P retention as estimated by shoot biomass production and total internal shoot P content. Shoot biomass production is commonly used as an index across crop species to measure tolerance to soil P-deficiency. Research

with cotton showed that decreases in soil P affected root hydraulic conductance, and this decreased conductance led to decreased water availability for leaf cells to expand during the daytime (Radin and Eidenbock 1984; Marschner 1995). Plants susceptible to soil P deficiency produced small leaves of a dark-green color because of small cell sizes while tolerant plants produce larger leaves with large cells (Hecht-Buchholz 1967; Marschner 1995). Overall, plants experiencing P-deficiency tend to experience a decrease in leaf expansion, leaf surface area, and number of leaves (Freeden 1989; Lynch 1991; Marschner 1995).

For this study, a methodology adapted from Johnson *et al*. (1994) was used to phenotype for tolerance to low soil P via shoot biomass measurements. Cowpea plants were grown in silica sand and watered with nutrient solutions of different P treatments. Various methods for using silica sand watered with nutrient solution as a means to grow and phenotype varieties for tolerance to P-deficiency have been employed in other crop species, e.g. white lupin (Johnson *et al*. 1994; Schulze *et al*. 2006), common bean (Yan *et al*. 1996; Hernández *et al*. 2007), blueberries (Yang and Goulart 1997), and peanuts (Wissuwa and Ae 2001). Also, silica sand mixed with alumina buffer and watered with nutrient solution has been used to phenotype for P-deficiency tolerance in tomato (Coltman *et al*. 1982 and Coltman *et al*. 1987), common bean (Lynch *et al*. 1990), and maize (da Silva *et al*. 1992). The results of Coltman *et al*. (1982) showed that shoot growth responded directly to the availability of P in the growing medium. The sand culture of Johnson *et al*. (1994) does not include alumina in the system but does display the same response of shoot growth to P availability in sand culture.

Via phenotyping in silica sand with nutrients added, the purpose of this study is to identify cowpea varieties with high tolerance to low soil P and elucidate the mechanism of tolerance by analyzing root biomass, root image, shoot internal P, root internal P, and seed P results. Shoot internal P content can serve as an indicator of a variety's tolerance to low soil P because vegetative nutrients are commonly mobilized to produce seed after flowering (Marschner 1995). For example, high leaf P has previously been linked to high seed production in barley (Saarela 1990). Also, a high shoot P, if shoot biomass is also high, indicates that a variety efficiently retained and recycled P while maximizing shoot production. Root biomass, root imaging, and internal root P content measurements of harvested plants will elucidate whether cowpea varieties were mobilizing P to the roots and increasing root production as mechanisms for tolerance.

Preliminary phenotyping results conducted for this study gave rise to interest in seed P as a key source of tolerance because of apparent associations between a large seed size and tolerance to P-deficiency. Seed P as a potential source of tolerance has been shown in other crop species (Bolland and Baker 1989; Riley *et al*. 1993; Marschner 1995; Yan *et al*. 1995; Liao and Yan 1999). These studies have noted a high total seed P can lead to seedlings with a large root surface area so that P is quickly acquired for an early seedling growth advantage (Bolland and Baker 1989; Riley *et al*. 1993; Marschner 1995; Yan *et al*. 1995). In addition, a study by Liao and Yan (1999) suggested that large seeds in common bean not only conferred an advantage because their seedlings have large root surface areas, but also because their seedlings have large leaf surface areas from large cells. Large leaf and root surface areas under normal nutrient conditions can

be physiological inefficient but during nutrient deficiencies are advantageous because of a larger surface area for absorption of light energy and soil nutrients.

This study was supplemented by a second smaller study to further investigate the effect of seed P on P-deficiency tolerance. The effect of seed P was analyzed via removal of cotyledons during early seedling growth. Cotyledons are sites of nutrient storage from the seed, and seedlings uptake cotyledon nutrients for growth before roots are fully formed and can uptake nutrients. In the literature, cotyledon removal has been previously used in bean (Hernández *et al*. 2007) and peanuts (Wissuwa and Ae 2001) to minimize the effect of seed P when phenotyping crop varieties for tolerance to P-deficiency.

2.2. Materials and Methods

2.2.1. *Phenotyping by sand culture*

Texas A&M University greenhouse facilities were used to grow cowpea varieties in a controlled environment. Greenhouse conditions were at 27 ºC daytime temperature and 23 ºC nighttime temperature. A daily photoperiod of 14 hours was applied by supplementing natural light with artificial lights if natural light reached below 700 W/m^2. One hundred and twenty one-gallon pots (cylindrical shape, 14.5 cm diameter by 16.5 cm height) were prepared for screening under three different P treatments: no P, low P (1.5 mg/kg P), and normal P (30 mg/kg P). Pots were lined with landscaping material cut into 18 in x 18 in (45.72 cm x 45.72 cm) pieces. Lined pots were filled with "Kosse White" silica sand (U.S. Silica, Kosse, TX), which ranges in particle size from

roughly 0.27 to 0.95 mm. Lining the pots ensured sand would not escape through pot drain holes while water or nutrient solution could still flow out. The pots were arranged in a randomized complete block design (RCBD) in the greenhouse with five replications of each variety and P treatment.

Eight cowpea varieties of either Nigerian or American origin (Table 1) were planted with Royal Peat Legume Seed Inoculant (Becker Underwood, St. Joseph, MO). in 15 replications in the greenhouse. Varieties chosen were based on preliminary phenotyping that had been conducted. All seeds planted were harvested from previous plantings of these varieties at Texas A&M University greenhouses and were not directly from external sources. Two seeds of a variety were planted in a pot and later thinned to one seedling at 10 days after planting (DAP).

Table 1 Cowpea varieties and their country of origin

Cultivar	Origin Country
Aloka	Nigeria
Big John	United States of America
CB-46	United States of America
Dan Ila	Nigeria
Golden Eye Cream	United States of America
IT97K-1069-6	IITA, Nigeria
IT98K-476-8	IITA, Nigeria
TX2028-1-3-1	United States of America

For each variety, 5 replications were watered with no P nutrient solution, 5 replications were watered with low P nutrient solution, and 5 replications were watered with normal P nutrient solution. Nutrient solutions were modified from Johnson *et al.* (1994) and created with reverse osmosis (RO) water. The composition of nutrients is shown in Table 2. The no P treatment did not contain $Ca(H_2PO_4)_2$, and the low and normal P treatments varied in $Ca(H_2PO_4)_2$ according to concentrations shown in Table 2. Pots were watered with nutrient solutions (pH adjusted to 6.5) according to Table 3. At 32 DAP pots that had previously received a full P treatment were watered with an additional 250 mL RO water to prevent wilting since plants were significantly larger and had higher water needs.

Table 2 Nutrient concentrations added to reverse osmosis (RO) water for application to cowpea varieties in sand culture

Nutrient	Molar concentration
KNO_3	3.0 mM
$Ca(NO_3)_2$	2.5 mM
$MgSO_4$	1.0 mM
FeEDTA	12.0 µM
$MnCl_2$	4.0 µM
H_3BO_3	22.0 µM
$ZnSO_4$	0.4 µM
$NaMoO_4$	0.05 µM
$CuSO_4$	1.6 µM
$Ca(H_2PO_4)_2$ (low P)	25.0 µM
$Ca(H_2PO_4)_2$ (normal P)	0.5 mM

Table 3 Timeline and quantity of nutrient solution applied per pot to phenotype cowpea varieties. Also indicated is the number of micrograms of each nutrient applied per pot

Days after planting (DAP)	Nutrient solution applied (mL)	KNO_3 (µg)	$Ca(NO_3)_2$ (µg)	$MgSO_4$ (µg)	FeEDTA (µg)	$MnCl_2$ (µg)	H_3BO_3 (µg)	$ZnSO_4$ (µg)	$NaMoO_4$ (µg)	$CuSO_4$ (µg)	$Ca(H_2PO_4)_2$ - low P (µg)	$Ca(H_2PO_4)_2$ - normal P (µg)
0	800	12135.0	16412.8	4816.0	176.3	20.2	54.4	25.9	0.3	10.2	234.2	4682.2
10	400	6067.5	8206.4	2408.0	88.2	10.1	27.2	13.0	0.2	5.1	117.1	2341.1
15	400	RO water without nutrients										
21	400	6067.5	8206.4	2408.0	88.2	10.1	27.2	13.0	0.2	5.1	117.1	2341.1
28	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
35	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
40	500	7584.4	10258.0	3010.0	110.2 1	12.6	34.0	16.2	0.2	6.4	146.4	2926.4

Each variety was uprooted with all shoots and roots left intact at the first sign of budding: TX2028-1-3-1 and Golden Eye Cream at 33 DAP; CB-46 and Aloka at 34 DAP; Dan Ila at 39 DAP; and IT97K-1069-6, Big John, and IT98K-476-8 at 45 DAP. Roots were washed with RO water and photographed with their shoots by placing the roots in a tray with one inch water depth to disperse the roots into a photographable array. An example photograph is given in Fig. 1. Roots were separated from the shoots at the crown for drying. Both shoots and roots were dried overnight at 75 ºC.

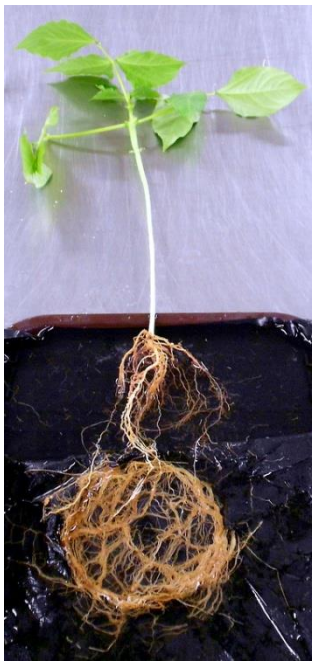

Fig. 1 Image of the cowpea variety Golden Eye Cream and its roots dispersed in water after growth in a low P sand culture system for 33 days

After being dried overnight, the masses of shoots and roots were recorded. The internal P concentration (mg/kg) of shoots and roots were then determined via inductively coupled plasma (ICP) analysis of a nitric acid digest (Isaac and Johnson 1980; Havlin and Soltanpour 1989). Total P content in shoot and roots samples was determined by multiplying the mass by the internal P concentration. Root image area was determined using the image analysis software GiA Roots (Galkovskyi *et al*. 2012).

Variety tolerance was determined by a P susceptibility index (PSI). The PSI was calculated as

$$PSI = \frac{1 - [Y_1 / Y]}{1 - [X_1 / X]}$$

where: Y_1 is the average dry shoot biomass in the low P treatment for the variety of interest; Y is the average dry shoot biomass in the low P treatment for all varieties; X_1 is the average dry shoot biomass in the normal P treatment for the variety of interest; and X is the average dry shoot biomass in the low P treatment for all varieties.

2.2.2. *Seed P measurements*

Seventy seeds of each variety were ground to fine powder and separated into subsamples for measurement of P concentration via ICP analysis of a nitric acid digest (Isaac and Johnson 1980; Havlin and Soltanpour 1989). Known average seed weights were then used to calculate the average total P in each variety's seed.

2.2.3. *Cotyledon removal study*

Texas A&M University greenhouse facilities were used to perform studies on the effect of seed P on a cowpea variety's growth in P-deficient soils. A second sand culture

study with a low P treatment was created in which cotyledons were removed from cowpea seedlings at 6, 7, 8, 9, and 10 days after planting (DAP). Also, a control in which cotyledons were not removed was included. The seedlings were allowed to continue growing until 35 DAP.

Thirty-six one-gallon pots were prepared according to the pot preparation method described in the section above. Six varieties were planted: Aloka, Golden Eye Cream, IT97K-1069-6, IT98K-1092-1, IT98K-476-8, and TX2028-1-3-1. IT98K-1092-1 was not a variety included in the first study, but preliminary phenotyping results of this Nigerian variety have shown it to be susceptible to low P treatments.

Each variety was planted in seven pots, and four seeds per pot were planted with Royal Peat Legume Seed Inoculant (Becker Underwood, St. Joseph, MO). One pot of each variety had its cotyledons removed at each of the following days: 6, 7, 8, 9, and 10 DAP. Fig. 2 shows sample images of how seedlings look at 6, 8, and 10 DAP when cotyledons were removed. Also, one pot for each variety did not have its cotyledons removed. At 6 DAP, seedlings had just emerged and the cotyledons were still large and smooth. By 10 DAP, cotyledons were small, shriveled, and about to abscise. Testing the removal of cotyledons at different DAP helped with detecting the effect cotyledon nutrients were having later on plant growth. Such knowledge was of interest to help discern the effect seed P has on cowpea's tolerance to soils or media deficient in P.

At 35 DAP, plants were harvested, and their roots were washed to remove the sand. Shoots were separated from roots at the crown, and then both shoots and roots were dried overnight at 75°C.

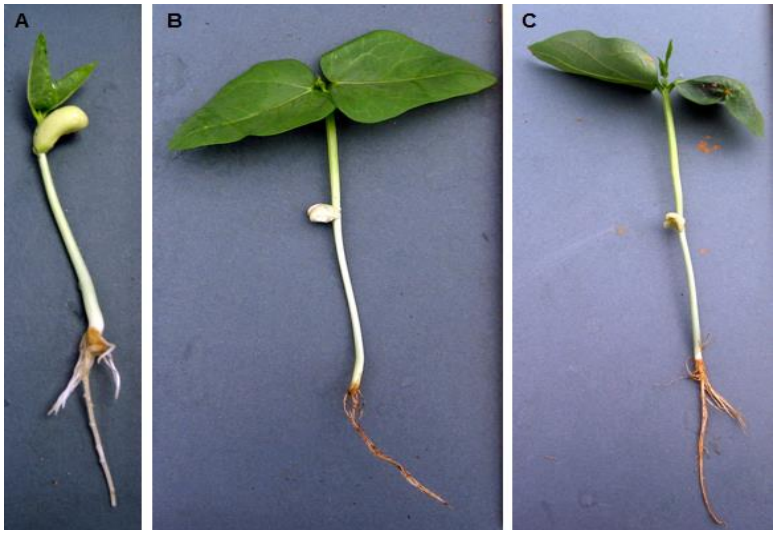

Fig. 2 Cowpea seedlings at 6 (A), 8 (B), and 10 (C) DAP. Photos show the growth stages of seedlings when their cotyledons were removed

2.3. Results

2.3.1. *Sand culture shoot biomass*

Graphs and images of shoot biomass results from the sand culture experiment are shown in Figs. 3 and 4 respectively. *T*-test comparisons of mean values are also given for each P treatment within a variety (Fig. 3). These results suggest Aloka and Dan Ila are susceptible varieties since shoot biomass is significantly lower under a low P treatment than a normal P treatment. For all varieties, shoot biomass when no external P added was lowest.

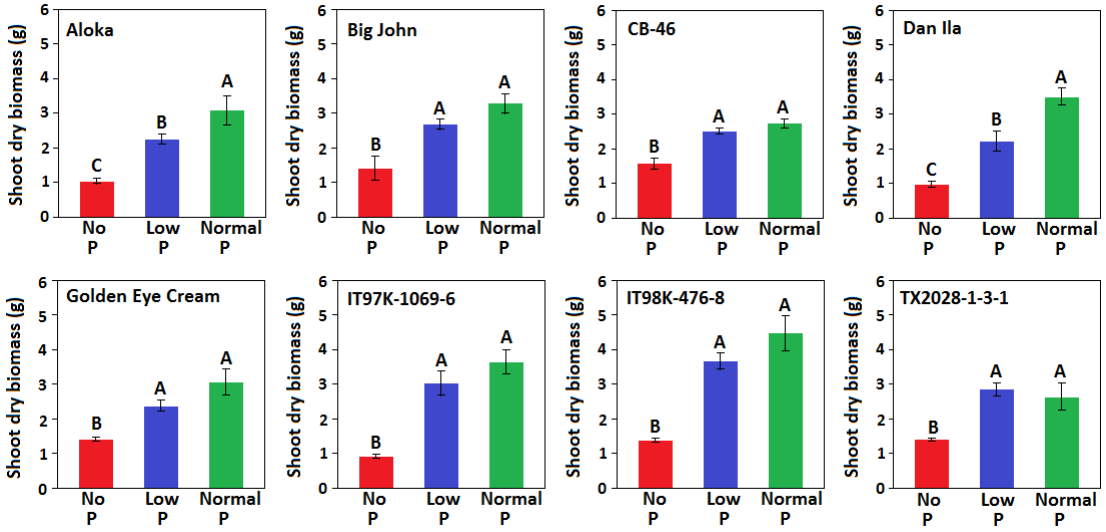

Fig. 3 Dry shoot biomass results for cowpea varieties grown in sand culture with three different P treatments. For each P treatment within a variety, *t*-test comparisons of mean values ($p = 0.05$) are included. Error bars represent standard error. Results indicate Aloka and Dan Ila as susceptible with shoot biomass production significantly lower under a low P treatment than a normal P treatment. For all varieties, shoot biomass was lowest when no P was added

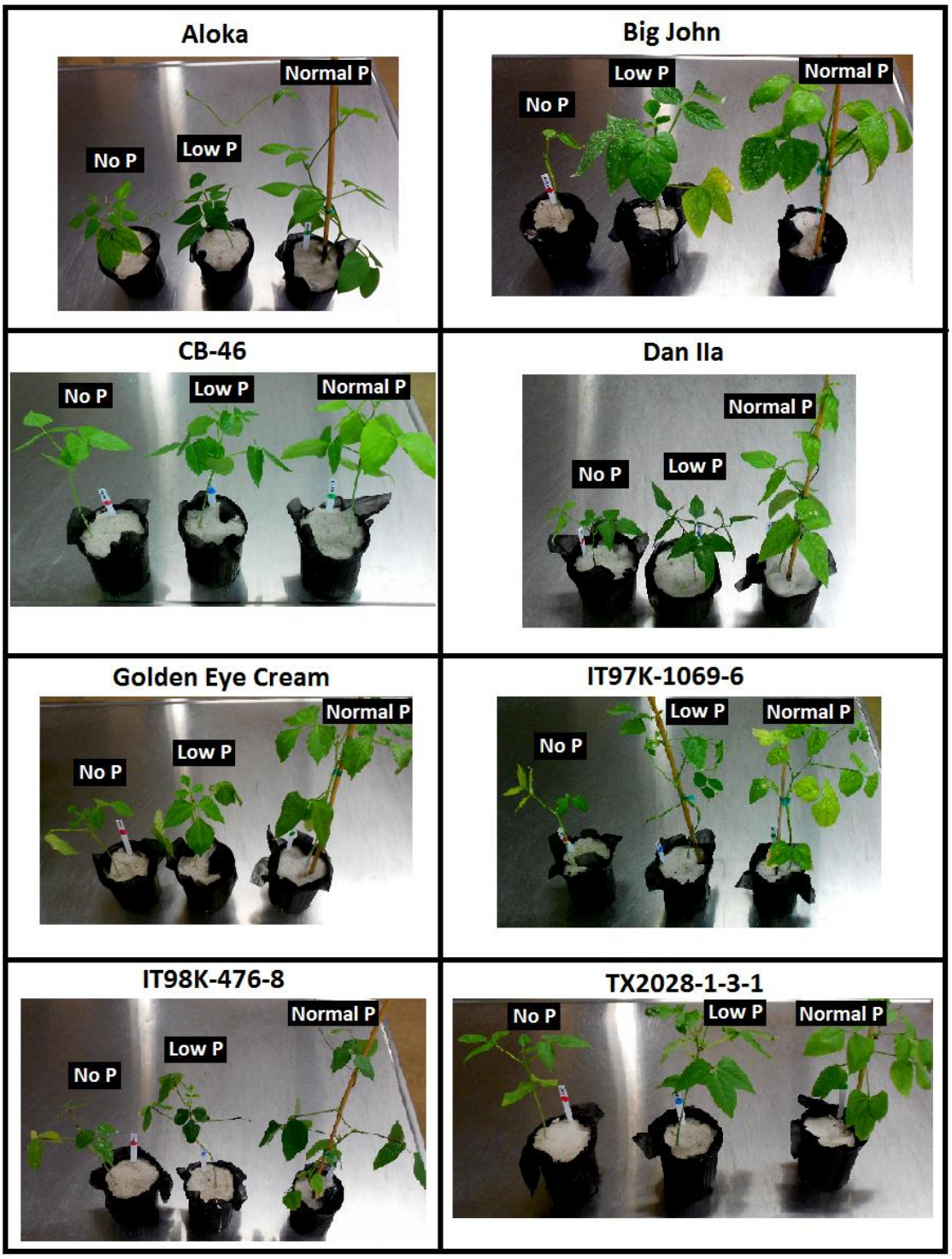

Fig. 4 Images of shoots before uprooting of cowpea varieties grown in sand culture with three different P treatments

PSI calculations for each variety are shown in Table 4. PSI calculations confirm comparisons of mean *t*-test results. Both Aloka and Dan Ila have high PSI values of 1.756 and 1.992, respectively, indicating susceptibility to P-deficiency. PSI values between 0 and 1.1, found in Big John, CB-46, Golden Eye Cream, IT98K-476-8, and IT97K-1069-6, indicate moderate to high tolerance to P-deficiency. The negative PSI value for TX2028-1-3-1 indicated that shoot growth under low P was higher than in normal P which would likely change if a more intense P-deficiency treatment had been applied.

Table 4 PSI calculations and results for each cowpea variety. PSI values greater than 1.1 indicate susceptibility to P-deficiency. Y_1 is the average dry shoot biomass in the low P treatment for the variety of interest; Y is the average dry shoot biomass in the low P treatment for all varieties; X_1 is the average dry shoot biomass in the normal P treatment for the variety of interest; and X is the average dry shoot biomass in the low P treatment for all varieties

Variety	Y_1	Y	X_1	X	PSI
Aloka	2.09978	3.09392	2.67939	3.27942	1.75617
Big John	2.68546	3.29054	2.67939	3.27942	1.00502
CB-46	2.55118	2.74194	2.67939	3.27942	0.38024
Dan Ila	2.23732	3.52018	2.67939	3.27942	1.99178
Golden Eye Cream	2.3894	2.81484	2.67939	3.27942	0.82606
IT98K-476-8	3.66864	4.479025	2.67939	3.27942	0.98886
IT97K-1069-6	2.9438	3.6475	2.67939	3.27942	1.05443
TX2028-1-3-1	2.85956	2.6474	2.67939	3.27942	-0.438

2.3.2. *Sand culture root biomass and shoot:root ratios*

The root biomass results from the sand culture experiment are shown in Fig. 5. *T*-test comparisons of mean values are also given for each P treatment within a variety (Fig. 5). Dry root biomass results indicate that some varieties – Aloka, Big John, CB-46, and TX2028-1-3-1 - had significantly higher root biomass under a low P treatment than under a normal P treatment. For other varieties, root biomass production under low P and normal P treatments were not significantly different. For all varieties, root biomass values were lowest when no P was added. These results suggest that in some varieties there is an enhanced root growth response to decreased P availability for foraging P.

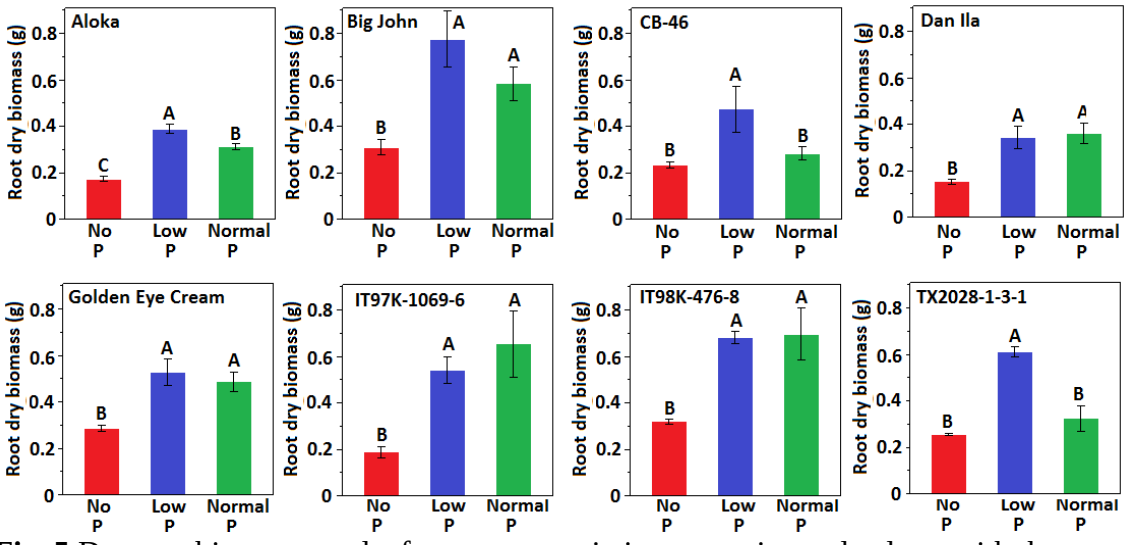

Fig. 5 Dry root biomass results for cowpea varieties grown in sand culture with three different P treatments. For each P treatment within a variety, *t*-test comparisons of mean values ($p = 0.05$) are included. Error bars represent standard error. Results indicate Aloka, CB-46, and TX2028-1-3-1 had significantly higher root production under a low P treatment than under a normal P treatment. For other varieties, root biomass production under low P and normal P treatments were not significantly different. For all varieties, root biomass with no P added was lowest

PSI values (not shown) for root biomass were not a good indicator of susceptibility because some varieties responded to the low P treatment by increasing root growth relative to the normal P treatment.

Table 5 shows the ratio of the shoot to root biomass results for each variety and treatment. All varieties experienced a decrease in shoot:root ratio from the normal to low P treatment.

Table 5 Ratio of shoot to root biomass for each cowpea variety and P treatment

	P treatment		
	No P	Low P	Normal P
Aloka	6.038309	5.498546	9.938036
Big John	4.424531	3.692835	5.828825
CB-46	6.738988	6.156001	10.02707
Dan Ila	6.457815	6.620149	10.32755
Golden Eye Cream	4.98461	4.715825	5.974959
IT97K-1069-6	5.062333	5.720205	7.322881
IT98K-476-8	4.351261	5.42007	6.638518
TX2028-1-3-1	5.532255	4.674332	8.253381

2.3.3. *Sand culture root imaging*

Root imaging results, shown in Fig. 6, deviate from root biomass results as indicated by *t*-test comparisons of mean. For Aloka, Dan Ila, IT98K-476-8, Golden Eye Cream, and TX2028-1-3-1, roots in the low P treatment had the greatest area relative to other P treatments according to comparisons of mean *t*-tests. For Big John, there were no significant differences in root area among any of the P treatments. For IT97K-1069-6

and CB-46, the low P treatment roots had the highest area relative to the normal P treatment but not the no P treatment. These results suggest again a foraging response through root growth by many varieties to decreased P availability.

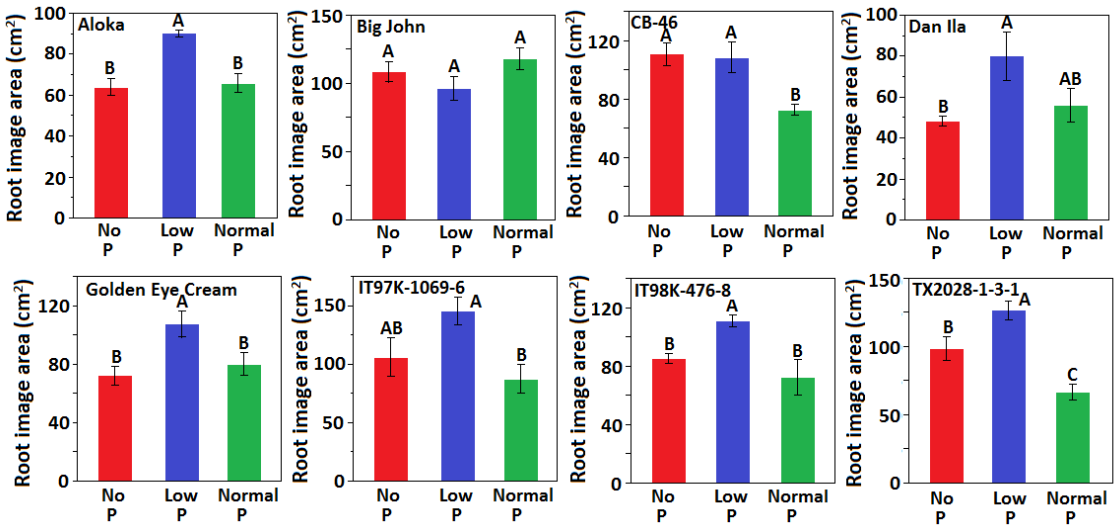

Fig. 6 Root image area results for cowpea varieties grown in sand culture with three different P treatments. Error bars represent standard error. Results from *t*-test comparisons of mean ($p = 0.05$) indicate Aloka, Golden Eye Cream, IT98K-476-8, and TX2028-1-3-1 had significantly higher root production under low P than under normal P treatments. For other varieties, root production under low P and normal P treatments were not significantly different. For only one variety, Big John, no significant differences in root image results for any of the P treatments was found. For IT97K-1069-6 and CB-46, root production under the low P treatment was not significantly higher than under the no P treatment

2.3.4. *Sand culture shoot internal P and original seed P*

Internal P content comparisons were made across varieties instead of within varieties for each P treatment. Comparisons of mean P content within a variety for each P treatment were not of interest because for all samples the internal P content increased proportionally with the amount of external P applied. For example, all plants under a normal P treatment experienced an approximate 8 to 12 fold increase in internal P content relative to plants under a low P treatment that is not reflected in shoot biomass growth. The results for P concentration (mg/kg) and total internal P content (mg) are shown in Fig. 7 with *t*-test comparisons of mean.

These internal P results gave further insight into which varieties have high low P tolerance and can retain P in their system when it is minimally available. A desired variety can maximize shoot biomass growth by utilizing minimal P, thus retaining more P within the plant to later be allocated for seed production. Before flowering, plants commonly mobilize the nutrients they uptake to vegetative growth, but once flowering begins nutrients are mobilized away from vegetative tissue and to the seed (Marschner 1995). Therefore, plants with high internal P before flowering will likely have the highest seed production and/or have seeds with higher P since more P is available from the vegetative tissue to be mobilized for seed production. Total shoot P results showed that IT98K-476-8 and IT97K-1069-6 retain the most P under a low P treatment. Big John and TX2028-1-3-1 showed intermediary uptake and retention of P under a low P treatment. These varieties would be the best candidate for high seed production with high seed P. The low shoot P retention for CB-46 and Golden Eye Cream under a low P

treatment indicates that these varieties do not have true low P tolerance and may be utilizing their seed P to increase shoot and root biomass growth under P deficiency. As noted in the introduction, large seeds with high seed P according to previous crop studies have been shown to confer a degree of tolerance to soil P-deficiency because of a large root and leaf surface areas produced by large seeds during early seedling growth that is largely advantageous when nutrient deficiencies exist.

Percent P in shoots (g P per 100 g shoot tissue) for optimal growth is considered to be in the range of 0.3-0.5 g P per 100 g shoot tissue during vegetative growth (Marschner 1995). One g P per 100 g shoot tissue or higher is considered to be entering into toxicity (Marschner 1995). The range of average shoot P concentrations under a low P treatment across varieties was from 901.4 mg/kg for CB-46 to 1139 mg/kg for Aloka, which are respectively equivalent to 0.09g P per 100 g shoot tissue to 0.11g P per 100 g shoot tissue. These levels indicate that all varieties in the low P treatment still had deficient shoot P content though some varieties experienced shoot biomass growth comparable to growth under the normal P treatment. The range of average shoot P concentrations under a normal P treatment across varieties was from 7621.2 mg/kg for Dan Ila to 10950.4 mg/kg for TX2028-1-3-1, which are respectively equivalent to 0.76g P per 100 g shoot tissue to 1.09g P per 100 g shoot tissue. These levels indicate that all varieties in the normal P treatment had more than adequate shoot P content and some varieties may have been on the verge of reaching P-toxicity.

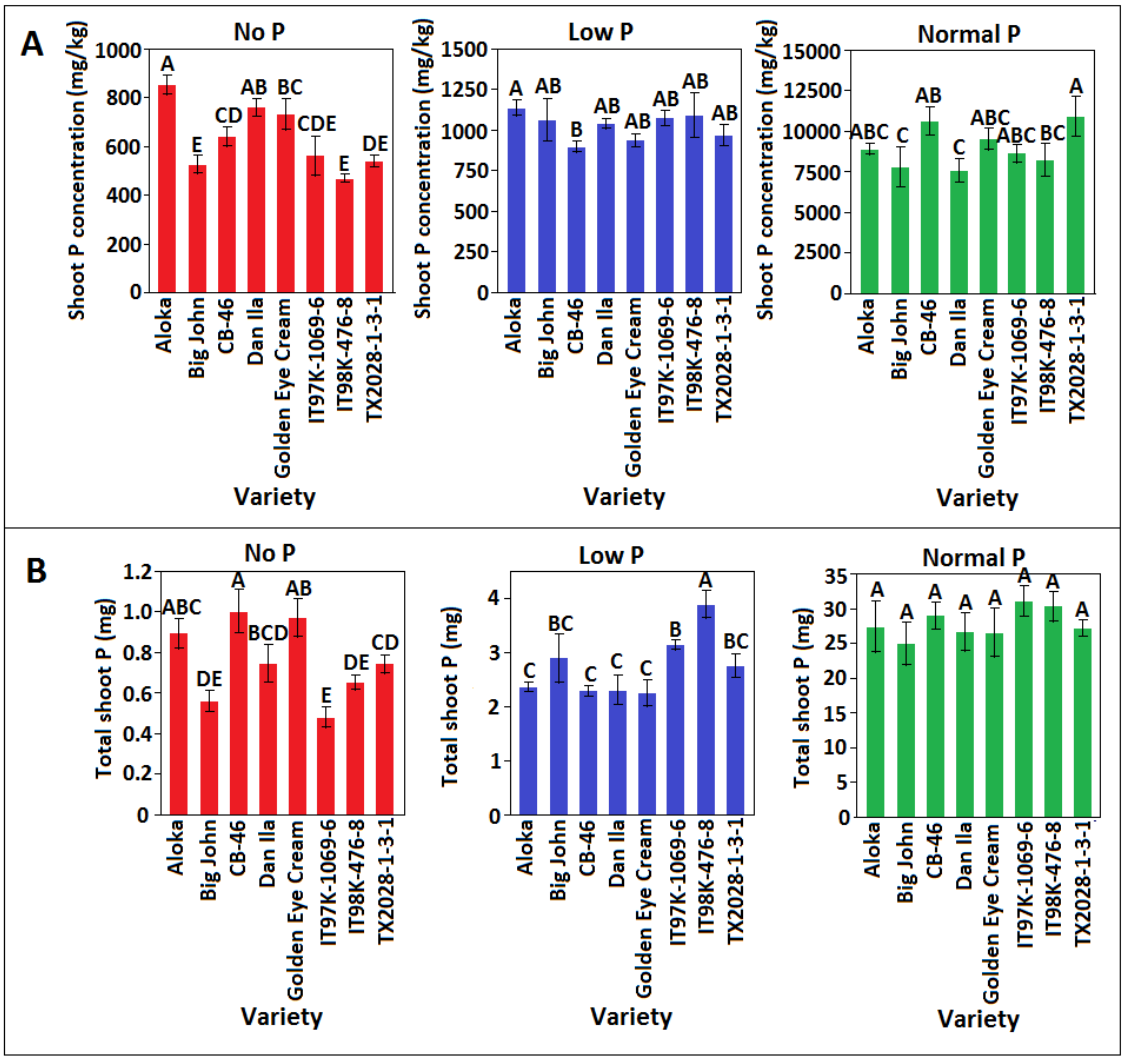

Fig. 7 Shoot internal P results for cowpea varieties grown in sand culture with three different P treatments. *T*-test comparisons of mean (p = 0.05) are also shown. Error bars represent standard error. Shoot P concentration (mg/kg) (A) results were measured directly from shoot samples. Total shoot P (mg) (B) were calculated by multiplying the shoot P concentration by the shoot dry biomass. Across all varieties there was a multi-fold increase in shoot P concentration and total P with an increase in externally applied P

Table 6 shows the results for each variety's initial seed P content and for the shoot biomass, internal P concentration, and total P content when no external P is applied. Making comparisons of these values gives further insight into whether a variety's tolerance is through seed P or a different low P tolerance mechanism. Big John, CB-46 and Golden Eye Cream had high initial seed P content. Results suggest that high initial seed P for Big John, CB-46 and Golden Eye Cream may confer an early vigor growth advantage. This early vigor may continue to have a positive effect in later stages as a variety experiences P-deficiency stress.

Comparisons between seed P and shoot biomass production when no external P is applied are of interest since seed P becomes the only P source for shoot biomass production. Aloka, Dan Ila, and IT97K-1069-6 had low shoot biomass production compared to other varieties when no external P was applied. These varieties also have the three lowest initial seed P contents. The higher shoot biomass of Big John, CB-46, and Golden Eye Cream may be interpreted as a result of high original seed P content. The higher shoot biomass of IT98K-476-8 and TX2028-1-3-1 may be from an ability to maximize growth with minimal P. Also of note is that TX2028-1-3-1 is an early maturing 60-day variety, and thus, the variety may just be displaying early maturity vigor. IT98K-476-8 as a low P tolerant variety is further discussed in section 2.3.5.

Table 6 The original seed P content for each variety and the shoot biomass, shoot P concentration, and total shoot P content for each variety when no external P is applied. The high seed P content of Aloka, Big John, and Golden Eye Cream may help confer tolerance to P-deficiency. When no external P is applied, Aloka, Dan Ila, and IT97K-1069-6 experience a drop in shoot biomass production relative to other varieties

Variety	Single Seed P (mg)	Mean Shoot Biomass (g)	Mean Shoot P Concentration (mg/kg)	Mean Total Shoot P (mg)
Aloka	0.568	1.048	855.4	0.896
Big John	1.471	1.422	527.3	0.564
CB-46	0.934	1.581	640.4	1.006
Dan Ila	0.498	0.993	761.0	0.748
Golden Eye Cream	0.822	1.421	681.5	0.972
IT97K-1069-6	0.721	0.916	563.0	0.4816
IT98K-476-8	0.728	1.390	470.4	0.653
TX2028-1-3-1	0.729	1.410	541.8	0.745

2.3.5. *Sand culture root internal P*

Comparisons of mean *t*-tests were performed on root P concentration and total P results to determine the statistical significance of differences across varieties for each P treatment (Fig. 8). These internal P results gave further insight into which varieties were tolerant to the low P treatment by actively uptaking P when it is minimally available. From the P concentration results, significant differences among varieties exist but are difficult to interpret. From the total P results, it appears Big John and IT98K-476-8 are uptaking more P into their roots when a low P treatment is applied followed by IT97K-1069-6, TX2028-1-3-1, and Golden Eye Cream. These results combined with the total shoot P results under a low P treatment suggest that tolerant varieties – IT98K-476-8, IT97K-1069-6, Big John, and TX2028-1-3-1 - are uptaking and/or retaining more P

when it is low in availability. Big John, CB-46, and Golden Eye Cream have a high seed P content lending to partial tolerance as well.

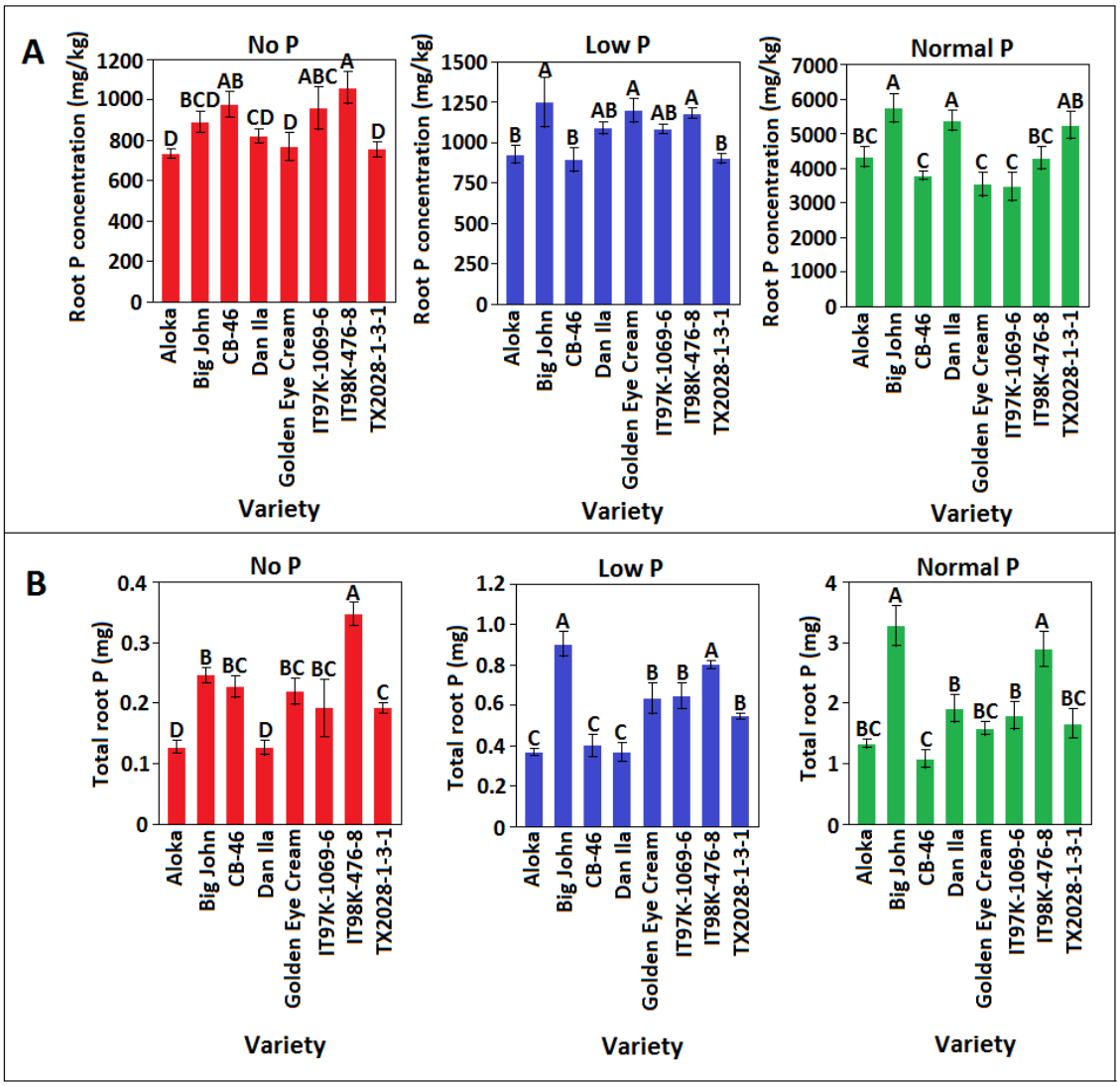

Fig. 8 Root internal P results for cowpea varieties grown in sand culture with three different P treatments. *T*-test comparisons of mean (p = 0.05) are also shown. Error bars represent standard error. Root P concentration (mg/kg) (A) results were measured directly from root samples. Total root P (mg) (B) were calculated by multiplying the root P concentration by the root dry biomass. Across all varieties there was a multi-fold increase in root P concentration and total P with an increase in externally applied P

2.3.6. *P utilization and P retention scores*

P utilization (PU) score was calculated as the mean shoot dry biomass (mg) divided by the mean plant P (mg). A high PU score would reflect a high shoot biomass obtained by utilizing a high portion of internal P so that a lower amount remains in the system. A P retention (PR) score, an inverse of PU, was calculated as the mean shoot dry biomass (mg) multiplied by the mean plant P (mg). A high PR would reflect a high shoot biomass obtained by efficiently utilizing minimal internal P so that a higher amounts remains in the system. Calculations are given in Table 7. Also worth noting is that Coltman *et al*. (1987) considered the PU score to be a calculation for PUE, but true PUE is not just high biomass production but also high retention of P within the plant. Considering that this internal P content is later allocated for seed production, not only a high shoot biomass but also a high internal shoot P is of interest.

Results show that when no P is added, the identified susceptible varieties have the lowest PU scores. Also, when no P is added, CB-46 and Golden Eye Cream have the highest PR scores, supporting these varieties as partially tolerant from seed P. When a low P treatment is added, CB-46, Golden Eye Cream, and TX2028-1-3-1 have slightly high PU scores, indicating these varieties may be displaying tolerance via high use of their internal P. Also, when a low P treatment is added, Aloka, CB-46, Dan Ila, and Golden Eye Cream have low PR scores, indicating they are not efficiently using or recycling P back into their system. IT98K-476-8 has a high PR score, indicating it is efficiently using and recycling P back into its system. PU and PR scores become less

relevant under normal P conditions since P is overabundant in the system. Interesting to note though is that IT98K-476-8 under normal P conditions has high PU and PR scores.

Table 7 Comparison of P utilization (PU) and P retention (PR) scores for each variety under each P treatment. Results largely support: Aloka and Dan Ila as susceptible varieties; CB-46 and Golden Eye Cream as partially tolerant varieties through a high utilization of seed P; IT98K-476-8 as tolerant through P retention. Results suggest under a low P treatment, CB-46, Golden Eye Cream, and possibly TX2028-1-3-1 are displaying tolerance through a high utilization of the P within the system

P treatment	Variety	Mean Shoot P (mg)	Mean Shoot Dry Biomass (mg)	P Utilization (PU) Score	P Retention (PR) score
No P	Aloka	0.8958	1048.20	1170.13	938.98
No P	Big John	0.5643	1421.70	2519.40	802.27
No P	CB-46	1.0058	1580.86	1571.74	1590.03
No P	Dan Ila	0.7484	992.70	1326.43	742.94
No P	Golden Eye Cream	0.9719	1814.24	1866.69	1763.26
No P	IT97K-1069-6	0.4816	916.075	1902.15	441.18
No P	IT98K-476-8	0.6528	1389.92	2129.17	907.34
No P	TX2028-1-3-1	0.7454	1410.48	1892.25	1051.37
Low P	Aloka	2.3703	2099.78	885.87	4977.11
Low P	Big John	2.9050	2685.46	924.43	7801.26
Low P	CB-46	2.2952	2551.18	1111.53	5855.47
Low P	Dan Ila	2.3148	2237.32	966.53	5178.95
Low P	Golden Eye Cream	2.2647	2389.40	1055.06	5411.27
Low P	IT97K-1069-6	3.1444	2943.80	936.20	9256.48
Low P	IT98K-476-8	3.9004	3668.64	940.58	14309.16
Low P	TX2028-1-3-1	2.7628	2859.56	1035.02	7900.39
Normal P	Aloka	27.4685	3093.92	112.64	84985.34
Normal P	Big John	25.0426	3290.54	131.40	82403.68
Normal P	CB-46	29.0538	2741.94	94.37	79663.78
Normal P	Dan Ila	26.6658	3520.18	132.01	93868.42
Normal P	Golden Eye Cream	26.5533	2814.84	106.01	74743.29
Normal P	IT97K-1069-6	31.1282	3647.50	117.18	113540.11
Normal P	IT98K-476-8	30.3508	4479.03	147.58	135941.99
Normal P	TX2028-1-3-1	27.2729	2647.40	97.07	72202.28

2.3.7. *Cotyledon removal effect*

Fig. 9 shows the shoot biomass results and the *t*-test comparisons of mean for the study on the effect of removing cotyledons, a source of plant nutrients from the seed, on plant growth in P-deficient conditions. Results indicate that a drop in shoot biomass production occurs if cotyledons were removed too early at 6 DAP, but if cotyledons were removed 7 DAP or later, plants were able to recover after losing their cotyledons as a nutrient source. The drop in shoot biomass production with removal of cotyledons at 6 DAP was noticeable across varieties though *t*-test comparisons of mean only identified the drop as significant for three of the six varieties. The results show that removal of the cotyledon as a P source during early seedling growth did not lead to significant intervarietal differences in shoot growth. Rather, it seems all varieties independent of original seed P content experienced similar effects from cotyledon removal at the different DAPs. If a high seed P content does have an effect on seedling vigor that leads to tolerance, which should have been detected in Golden Eye Cream, then the positive effect may be taking place during germination and pre-emergence from the soil.

Preliminary studies to this research on the effect of cotyledon removal on seedling growth had been done with P-deficient soil from Nacogdoches, TX. In those studies, the same results of cotyledon removal at 6 DAP but not later affecting plant growth were identified. The P content of cotyledons removed at each DAP in that study was calculated, and it was determined that cotyledons at 6 DAP had only about half the total P content of the original seed, indicating much of the seed P had already been utilized by 6 DAP. Unfortunately, 6 DAP is the first day seedlings are above soil with

leaves out so that cotyledons can be removed readily. Any beneficial effect a high seed P may confer for tolerance to P-deficiency likely occurs while the seedling is germinating and before emerging out of the soil and was not able to be investigated with this study.

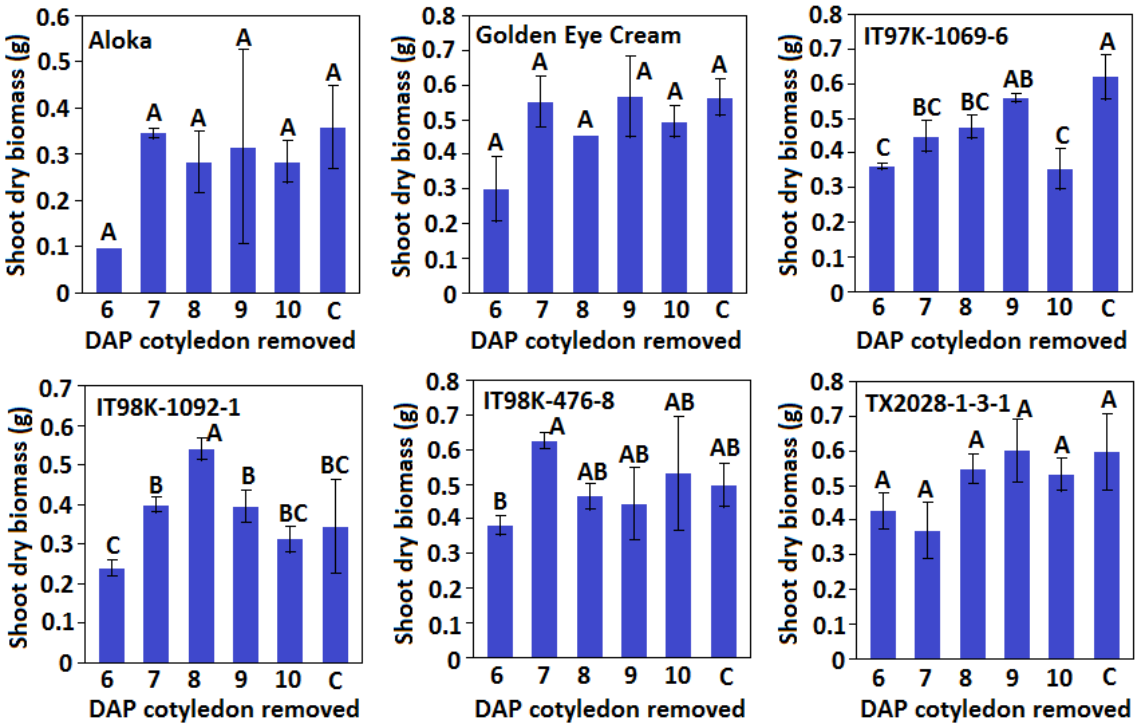

Fig. 9 Shoot dry biomass results for testing the effect of removing cotyledons, a nutrient source for early seedling growth from the seed, on cowpea growth in a low P treatment. *T*-test comparisons of mean (p = 0.05) are also shown. Error bars represent standard error. Cotyledons were removed at either 6, 7, 8, 9, or 10 DAP. Also included was a control with no cotyledons removed. The shoots were harvested for biomass at 35 DAP

Fig. 10 shows the root biomass results and the *t*-test comparisons of mean for the study. Results do not deviate far from shoot biomass results in that similar *t*-test comparisons of mean were obtained. Root growth along with shoot growth was stunted if cotyledons were removed early at 6 DAP.

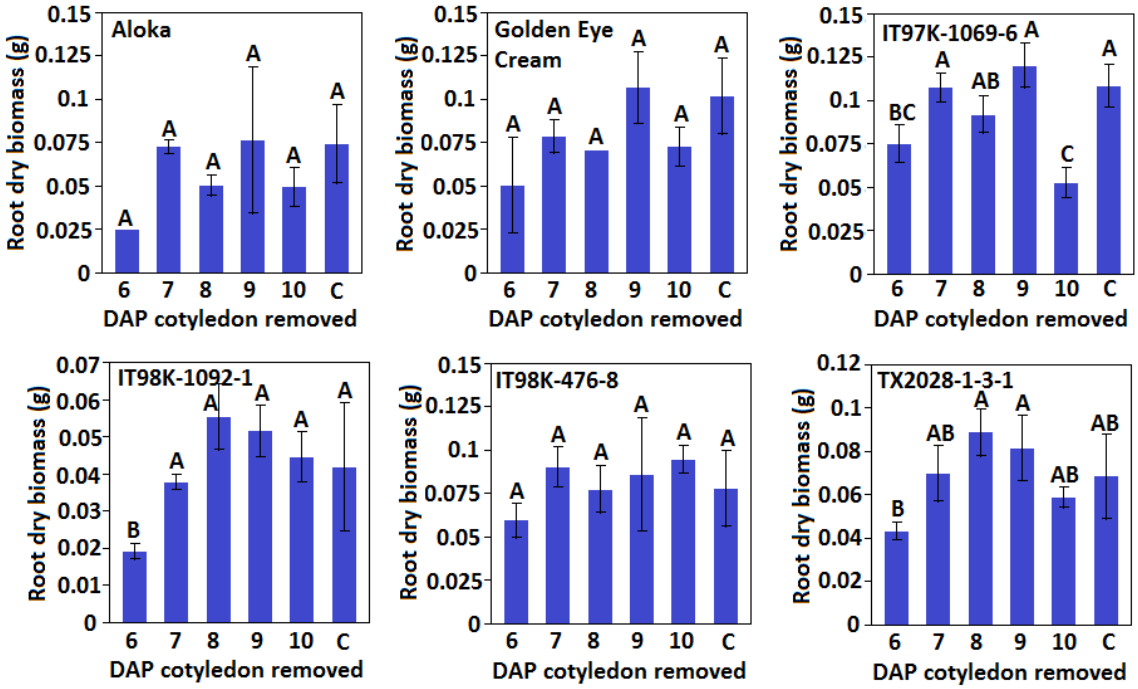

Fig. 10 Root dry biomass results for testing the effect of removing cotyledons, a nutrient source for early seedling growth from the seed, on cowpea growth in a low P treatment. *T*-test comparisons of mean ($p = 0.05$) are also shown. Error bars represent standard error. Cotyledons were removed at either 6, 7, 8, 9, or 10 DAP. Also included was a control with no cotyledons removed

2.4. Discussion

The cause for tolerance to soil nutrient deficiencies can vary among varieties within a crop species. The results of this study support enhanced root growth, high seed P, and P retention as low P tolerance mechanisms in cowpea as identified by studying six cowpea varieties previously identified as tolerant and two susceptible varieties.

The methodology adopted from Johnson *et al*. (1994) in which varieties were grown in silica sand-filled pots watered by nutrient solutions of different P treatments successfully phenotyped varieties for tolerance, particularly after calculating PSI values. This phenotyping method was also used for analyzing the heritability of low P tolerance and for mapping low P tolerance in cowpea (Sections 3 and 4).

Root mass and root imaging results from phenotyping showed that several cowpea varieties increased root production under a low P treatment relative to a normal P treatment as they foraged for P via enhanced root production. Other studies on crop tolerance to soil P deficiencies have commonly linked increased root production to tolerance (Khamis *et al*. 1990; Smith *et al*. 1990; Marschner 1995). If exposed to P-deficiency, plants start to partition photosynthates to the roots, and a decrease in shoot:root biomass ratio is commonly observed (Khamis *et al*. 1990; Marschner 1995). In *Stylosanthes hamata*, an increase in root production under P deficiency relative to under normal P was also observed because of translocation of P from the shoots to the roots (Smith *et al*. 1990). Many crop species' roots in P-deficient soils increase their root production by elongating their roots so that they are finer but longer, presumably for enhanced foraging of soils for nutrients (Marschner 1995). Also, proteoid root

production has several times been identified as a plant adaptation to P-deficiency (Marschner 1995). A decrease in shoot:root biomass ratio from a normal P treatment relative to a low P treatment is typically an indicator of tolerance to P-deficiency for a variety. However, in this study, known susceptible cowpea varieties Aloka and Dan Ila according to shoot biomass, root biomass, and root imaging results were among those to experience a decrease in shoot:root ratio under low P treatments relative to normal P treatments. These results suggest other mechanisms exist for conferring tolerance to P-deficiency in cowpea varieties in addition to increased root production.

Seed P was also investigated as a cause of P-deficiency tolerance. Three varieties Big John, CB-46, and Golden Eye Cream have high seed P content, and these three varieties also had high shoot biomass production under both no P and low P conditions. These results suggest that high seed P can be a source of early vigor for varieties that continues to have a positive effect if varieties start to experience P-deficiency stress. As suggested in other studies, high seed P seems to lead to large seedling root and leaf surface areas which are advantageous when soil nutrient deficiencies exist.

A study on removing cotyledons starting 6 DAP to test the effect of seed P on later plant growth in a low P treatment did not indicate any significant differences among varieties in response. Such results suggest that any benefit that high seed P has for a variety may be conferred very early during seed germination and prior to seedling leaves opening out of the cotyledons.

The main traits of interest for this study were P utilization (PU) and P retention (PR) reflected by total shoot biomass production and internal shoot P. The variety with

the highest ability for recycling and retaining P in its system while maximizing shoot growth was IT98K-476-8. IT98K-476-8 had high shoot biomass growth under both no P and low P treatments. Also, under the low P treatment, IT98K-476-8 retained a significantly high total amount of P in its biomass. When a PR score was calculated for varieties by multiplying shoot biomass production by shoot internal P, except when no P was added, IT98K-476-8 had the highest PR scores. These results suggest that the PR of IT98K-476-8 is not necessarily linked to recycling P from and back to the seed, but rather, IT98K-476-8 acquires external P efficiently and retains it within its system. These results confirm observations made by Saidu *et al*. (2011) that IT98K-476-8 is a low-P tolerant variety. Such characteristics for high uptake and retention of P by IT98K-476-8 make this variety as a good parent for breeding tolerant cowpea varieties for soils poor in P.

Under the no P treatment, CB-46 and Golden Eye Cream had the highest PR scores, suggesting these varieties are retaining their seed P content if stunted and not able to uptake any more P for growth. However, as soon as some external P is available, CB-46 and Golden Eye Cream appear to start using their seed P reserves to maximize shoot production. These results do leave some question as to how these varieties if utilizing their seed P reserves for growth under minimal external P application would be able to recycle P back into the next generation of seed.

Big John is suspected to have, like CB-46 and Golden Eye Cream, early growth vigor from high seed P content, but Big John uses up this seed P store more rapidly under the no P treatment than CB-46 and Golden Eye Cream. IT97K-1069-6, like

IT98K-476-8, is suspected to be efficient at taking up P when it is minimally available and recycling it while maximizing shoot growth. Big John and TX2028-1-3-1 may also be efficient in their P-uptake to maximize growth but do not retain this P as well in their system.

Overall, my results show that among cowpea varieties there are various physiological responses to P-deficiency stress that can lead to tolerance. For breeding purposes, a variety is desired that can efficiently forage and uptake soil P when it is limited in availability, recycle and retain this P in its system while maximizing shoot biomass production, and then return this P back into its seed for the next generation. Several varieties of this study displayed at least one but not all of these traits. If such a variety is to be bred, it is worth noting that there are other physiological mechanisms, especially related to roots, that can serve for enhancing P uptake, or really PAE, that were not examined in this study, such as mycorrhizal fungi associations, root surface anion exchange capacity, root organic acid exudation, and root hair production. If PU and PR, as focused on in this study, can be combined with PAE for crop varieties to be grown in P-deficient soils, then crop yields would greatly increase. Crops with PU, PR, and PAE are of high interest for future breeding of crop varieties for P-deficient soils, as P-reserves become increasingly depleted worldwide.

3. GENETIC CONTROL AND INHERITANCE OF TOLERANCE TO LOW PHOSPHORUS SOILS IN COWPEA

3.1. Introduction

Cowpea is the staple legume crop of Sub-Saharan West Africa where its leaves, pods, and seed are eaten by both people and livestock. However, the soils of West Africa are low in P, one of the three macronutrients plants require for normal growth and seed production. This soil fertility problem, along with other environmental stresses such as drought, insects, and disease pressure, causes decreased cowpea yield to approximately 240 kg/ha while trial tests predict a potential yield can reach ten times higher (Quin 1997, Chimphango *et al*. 2008).

Tolerant and partially tolerant cowpea varieties to low phosphorus soil conditions have been identified (Section 2). Thirty-five to forty day phenotyping for tolerance to P-defiency was developed and paved the way for this study of the genetic control and inheritance of tolerance to low P soils. Cowpea is a diploid self-pollinating species but is readily crossed through hand emasculation and pollination. To test the genetic effects and broad- and narrow-sense heritability of tolerance to low P soils, F_1, BC_1, and F_2 seeds were produced from two tolerant to susceptible crosses of cowpea's tolerance to low P soils. To further test heritability, F_1 seed was made from several more tolerant to susceptible crosses. Phenotyping this seed and analyzing the results can be used to define the genetic control and heritability of the trait which can be utilized for future breeding purposes and genetic marker identification.

Studies on the genetic control and heritability of P-deficiency tolerance in crop species have been previously conducted. Coltman *et al*. (1987) calculated the broad-sense heritability, P uptake (mg P/plant), and P utilization ratio (mg SDW/mg P) of tomato grown in low and high P sand-alumina cultures. Broad-sense heritability estimates were in the range of 61% to 67%, indicating important dominant genetic variance. Coltman *et al*. (1987) noted that, previously, dominant and epistatic variance had been identified as important for root development in low P conditions in beans (Fawole *et al*. 1982a) and in cucumbers (Ghaderi and Lower 1979) and for P-use efficiency (PUE) in low P conditions in beans (Fawole *et al*. 1982b). Da Silva *et al*. (1992) calculated the general and specific combining abilities of maize varieties grown in a low P sand-alumina culture. Their results indicated that in maize tolerance to low P was controlled by additive gene effects though dominance was also important. Araújo *et al*. (2005) calculated the additive effects, dominance effects, and broad-sense heritability for common bean grown in a sandy clay loam soil with limited P. Their results indicated broad-sense heritability estimates of 41% and 67% for shoot biomass in two separate experiments. They also estimated broad-sense heritability for several root traits (lateral root mass, root area, root mass) and for total P content to be largely in the 50% to 55% range. For these traits, they measured significant additive effects but not dominance effects. Parentoni *et al*. (2010) calculated the additive, dominance, and epistatic gene effects in maize grown in one low P field site and one high P field site. For grain yield and PAE, they discovered dominance effects followed by epistatic effects were more important than additive effects. However, they also noted under normal P these

dominance effects became less important. Ojo *et al*. (2007) calculated the heritability and genetic control of P-acquisition (PAE) in cowpea via uptake of rock phosphate (RP). For yield and PAE, their results also indicated significant dominance and epistatic effects. Broad-sense heritability was estimated to be 55.24%, but narrow-sense heritability was estimated to be rather low at 28.39%. They also calculated the heritability and genetic control of seed P concentration for their varieties segregating for PAE, and they noted significant additive, dominance, and epistatic effects. For seed P, high broad-sense heritability was estimated at 78.58% and high narrow-sense heritability was estimated at 50.57%. These previous studies suggest that, in cowpea, dominance effects are expected to be responsible for P-deficiency tolerance, and high broad-sense heritability is expected for tolerance. However, this study differs from that of Ojo *et al*. (2007) in that it focuses on low P tolerance in cowpea as determined by shoot biomass growth in a P-deficient sand culture while their studied focused on PAE as determined by yield in soils with RP.

In addition, this study will calculate the number of genes controlling low P tolerance in cowpea. Previous studies on the number of genes controlling nutrient use in crops have at times identified a single gene pair to be responsible, but often a complex gene system is responsible (Marschner 1995). In the study of PAE in cowpea by Ojo *et al*. (2007), they calculated a single gene effect as responsible for yield and possibly a single gene or multiple genes as responsible for seed P concentration. They suspected calculations were being biased toward single gene effects because of epistasis.

The results of this study on the heritability and genetic control of low P tolerance in cowpea can be compared to the results of the previous studies just described and others. These results provide the back drop for breeding and marker identification of low P tolerance in cowpea and other crop species.

3.2. Materials and Methods

3.2.1. *Phenotyping by sand culture progeny from crosses of IT98K-476-8 (tolerant) and Aloka (susceptible)*

Texas A&M University greenhouse facilities were used to grow cowpea varieties in a controlled environment. Greenhouse conditions were at 27 ºC daytime temperature and 23 ºC nighttime temperature. A daily photoperiod of 14 hours was applied by supplementing natural light with artificial lights if natural light reached below 700 W/m^2. Two-hundred and six one-gallon pots (cylindrical shape, 14.5 cm diameter by 16.5 cm height) were prepared for screening under a low P (1.5 mg/kg P) treatment. Pots were lined with landscaping material cut into 18 in x 18 in (45.72 cm x 45.72 cm) pieces. Lined pots were filled with "Kosse White" silica sand (U.S. Silica, Kosse, TX)), which ranges in particle size from roughly 0.27 to 0.95 mm. Lining the pots ensured sand would not escape through pot drain holes but nutrient solution could readily flow through. The pots were arranged in a randomized complete block design (RCBD) in the greenhouse. Two seeds per pot were planted and later thinned to one seedling per pot at 10 days after planting (DAP). Seeds were planted with Royal Peat Legume Seed Inoculant (Becker Underwood, St. Joseph, MO). A list of each variety and their progeny

planted is listed in Table 8. All seeds planted were harvested from previous plantings at Texas A&M University greenhouses and were not from external sources. IT98K-476-8 was a variety previously identified as tolerant to low P treatments, and likewise, Aloka was susceptible.

Table 8 Seed count for IT98K-476-8 and Aloka and their F_1, BC_1, and F_2 progeny planted and screened under a low P treatment in the greenhouse

Variety or Progeny	Number
IT98K-476-8 (tolerant)	20
Aloka (susceptible	19
F_1	13
BC_1 to IT98K-476-8	14
BC_1 to Aloka	16
F_2	122

Nutrient solutions were modified from Johnson *et al.* (1994) and created with reverse osmosis (RO) water. The nutrient solution composition is shown in Table 9. Pots were watered with nutrient solution (pH adjusted to 6.5) and RO water according to Table 10. All plants were uprooted with all shoots and roots left intact at 40 DAP when the first sign of budding occurred in Aloka. Roots were hand-washed and were separated from the shoots at the crown for drying. Both shoots and roots were dried overnight at 75 ^{o}C. After being dried overnight, the masses of shoots and roots were taken and recorded.

Table 9 Nutrient concentrations added to reverse osmosis (RO) water for application to IT98K-476-8 and Aloka and their F_1, BC_1, and F_2 progeny in sand culture

Nutrient	Molar concentration
KNO_3	3.0 mM
$Ca(NO_3)_2$	2.5 mM
$MgSO_4$	1.0 mM
FeEDTA	12.0 µM
$MnCl_2$	4.0 µM
H_3BO_3	22.0 µM
$ZnSO_4$	0.4 µM
$NaMoO_4$	0.05 µM
$CuSO_4$	1.6 µM
$Ca(H_2PO_4)_2$ (low P)	25.0 µM

Table 10 Timeline and quantity of nutrient solution applied per pot to phenotype IT98K-476-8 and Aloka and their F_1, BC_1, and F_2 progeny in sand culture. Also indicated is the number of micrograms of each nutrient applied per pot. One application did not contain any $Ca(H_2PO_4)_2$

Days after planting (DAP)	Nutrient solution applied (mL)	KNO_3 (µg)	$Ca(NO_3)_2$ (µg)	$MgSO_4$ (µg)	FeEDTA (µg)	$MnCl_2$ (µg)	H_3BO_3 (µg)	$ZnSO_4$ (µg)	$NaMoO_4$ (µg)	$CuSO_4$ (µg)	$Ca(H_2PO_4)_2$ - low P (µg)	$Ca(H_2PO_4)_2$ - normal P (µg)
0	500	RO water without nutrients										
5	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
9	250	RO water without nutrients										
12	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
16	250	RO water without nutrients										
19	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
26	250 (no P)	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	0	0
29	250	RO water without nutrients										
34	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2

3.2.2. *Phenotyping by sand culture progeny from crosses of Big John (tolerant) and Dan Ila (susceptible)*

To confirm results from the screening of IT98K-476-8, Aloka, and their progeny, a similar screen for Big John, Dan Ila, and their progeny was created. Big John is a tolerant variety to low P media conditions while Dan Ila is susceptible.

Texas A&M University greenhouse facilities were used to grow cowpea varieties in a controlled environment. Greenhouse conditions were at 27 ºC daytime temperature and 23 ºC nighttime temperature. A daily photoperiod of 14 hours was applied by supplementing natural light with artificial lights if natural light reached below 700 W/m^2. Sixty-two one-gallon pots (cylindrical shape, 14.5 cm diameter by 16.5 cm height) were prepared for screening. Pots were lined with landscaping material cut into 18 in x 18 in (45.72 cm x 45.72 cm) pieces. Lined pots were filled with "Kosse White" silica sand (U.S. Silica, Kosse, TX), which ranges in particle size from roughly 0.27 to 0.95 mm. Lining the pots ensured sand would not escape through pot drain holes but nutrient solution could readily flow through. The pots were arranged in a randomized complete block design (RCBD) in the greenhouse. Two seeds per pot were planted and later thinned to one seedling per pot at 10 DAP. Seeds were planted with Royal Peat Legume Seed Inoculant (Becker Underwood, St. Joseph, MO). A list of each variety and their progeny planted is listed in Table 11. All seeds planted were harvested from previous plantings at Texas A&M University greenhouses and were not from external sources.

Table 11 Seed count for Big John and Dan Ila and their F_1, BC_1, and F_2 seed planted and screened under a low P treatment in the greenhouse

Variety or Progeny	Number
Big John	4
Dan Ila	4
F_1	3
BC_1 to Big John	11
BC_1 to Dan Ila	8
F_2	32

Nutrient solutions were modified from Johnson *et al.* (1994) and created with reverse osmosis (RO) water according to concentrations shown in Table 9. Pots were watered with nutrient solution (pH adjusted to 6.5) and RO water as indicated in Table 12, a modified version of the sand culture screen described in Section 2. All plants were uprooted with all shoots and roots left intact at 32 DAP. Roots were hand-washed and were separated from the shoots at the crown for drying. Both shoots and roots were dried overnight at 75 ºC. After being dried overnight, the masses of shoots and roots were taken and recorded.

Table 12 Timeline and quantity of nutrient solution applied per pot to phenotype Big John and Dan Ila and their F_1, BC_1, and F_2 progeny in sand culture. Also indicated is the number of micrograms of each nutrient applied per pot. Three applications did not contain any $Ca(H_2PO_4)_2$

Days after planting (DAP)	Nutrient solution applied (mL)	KNO_3 (µg)	$Ca(NO_3)_2$ (µg)	$MgSO_4$ (µg)	FeEDTA (µg)	$MnCl_2$ (µg)	H_3BO_3 (µg)	$ZnSO_4$ (µg)	$NaMoO_4$ (µg)	$CuSO_4$ (µg)	$Ca(H_2PO_4)_2$ - low P (µg)	$Ca(H_2PO_4)_2$ - normal P (µg)
0	600	RO water without nutrients										
5	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
13	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
17	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
23	250 (no P)	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	0	0
25	250 (no P)	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	0	0
28	250 (no P)	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	0	0

3.2.3. *Phenotyping by sand culture other F_1 progeny*

After these screenings, a third screen of F_1 seed and parent variety seed from other crosses was conducted to further test the inheritance of the low P tolerance trait. Crosses of tolerant by susceptible, tolerant by semi-tolerant, and tolerant by tolerant were included. A control treatment of normal P was also included. Table 13 is a list of the F_1 seed planted and their numbers. All seeds planted were harvested from previous plantings at Texas A&M University greenhouses and were not from external sources.

Table 13 F_1 seed planted and screened under a low P treatment in the greenhouse

Variety or Progeny	Number
IT97K-1069-6 (tolerant) x Aloka (susceptible)	4
IT98K-476-8 (tolerant) x Dan Ila (susceptible)	3
Dan Ila (susceptible) x IT98K-476-8 (tolerant)	4
Aloka (susceptible) x Big John (tolerant)	4
IT98K-476-8 (tolerant) x Golden Eye Cream (semi-tolerant)	2
Big John (tolerant) x Golden Eye Cream (semi-tolerant)	4
CB-46 (semi-tolerant) x IT98K-476-8 (tolerant)	4
CB-46 (semi-tolerant) x Big John (tolerant)	4
Golden Eye Cream (semi-tolerant) x IT97K-1069-6 (tolerant)	4
Golden Eye Cream (semi-tolerant) x IT98K-476-8 (tolerant)	4
Golden Eye Cream (semi-tolerant) x Big John (tolerant)	4
IT97K-1069-6 (tolerant) x TX2028-1-3-1 (tolerant)	4
IT97K-1069-6 (tolerant) x IT98K-476-8 (tolerant)	4
IT97K-1069-6 (tolerant) x Big John	4
TX2028-1-3-1 (tolerant) x IT98K-476-8 (tolerant)	4

Nutrient solutions were modified from Johnson *et al.* (1994) and created with reverse osmosis (RO) water according to concentrations shown in Table 9. The control pots watered with a normal P treatment contained 0.5 mM $Ca(H_2PO_4)_2$ instead of 25.0 µM. Pots were watered with nutrient solution (pH adjusted to 6.5) and RO water according to Table 14, a modified version of the sand culture screen described in Section 2. At 37 DAP pots that had previously received a full P treatment were watered with an additional 250 mL RO water to prevent wilting since plants were significantly larger and had higher water needs. All plants were uprooted with all shoots and roots left intact at 43 DAP. Roots were hand-washed and were separated from the shoots at the crown for drying. Both shoots and roots were dried overnight at 75 ºC. After being dried overnight, the masses of shoots and roots were taken and recorded.

3.2.4. *Estimating genetic effects and heritability for the low P tolerance trait*

Statistical Analysis System (SAS) program (SAS, 1985) was used to perform generation means analysis on shoot biomass and root biomass results to determine additive, dominance, and epistatic effects. The generation means analysis used was proposed by Gamble (1962), and in it the following equations and terms are used:

$P_1 = m + a + aa$

$P_2 = m – a + aa$

$F_1 = m + d + dd$

$F_2 = m + 0.5d + 0.25dd$

$BC_1P_1 = m + 0.5a + 0.5d + 0.25aa + 0.25ad + 0.25dd$

$BC_1P_2 = m – 0.5a + 0.5d + 0.25aa + 0.25ad + 0.25dd,$

where m is the overall mean, a is the additive effect, d is the dominance effect, aa is the additive-by-additive effect, ad is the additive-by-dominance effect, and dd is the dominance-by-dominance effect.

SAS output results, in addition to additive, dominance, and epistatic effects, also gave the variance for each parent and progeny type. These variances were used to calculate additive, dominance, and environmental variance for shoot biomass and root biomass results, which then were used to calculate narrow- and broad-sense heritability of low P tolerance. Calculations were adapted from Warner (1952). The variance calculations are as follows:

$V_E = [(n_1 - 1)\ VarP_1 + (n_2 - 1)\ VarP_2 + (n_3 - 1)\ VarF_1]/(n_1 + n_2 + n_3 - 3)$

$V_A = 2*VarF_2 - VarBC_1 - VarBC_2$

$V_D = VarBC_1 + VarBC_2 - VarF_2 - V_E$

The heritability calculations are as follows:

$h^2_B = V_G / (V_G + V_E)$

$h^2_N = V_A / (V_G + V_E)$,

where V_G is V_A and V_D added together.

The number of genes involved with low P tolerance and with root biomass production was also estimated with the calculation: $n = (P_1 - P_2)^2 / 8*Var_AF_2$, where P_1 and P_2 are the mean values of each parent in the cross and Var_AF_2 is the additive genetic variance in the F_2 (Castle 1921; Lande 1981;Wright 1968; Zeng *et al*. 1990).

Table 14 Timeline and quantity of nutrient solution applied per pot to phenotype other F_1 progeny in sand culture. Also indicated is the number of micrograms of each nutrient applied per pot. One application did not contain any $Ca(H_2PO_4)_2$

Days after planting (DAP)	Nutrient solution applied (mL)	KNO_3 (µg)	$Ca(NO_3)_2$ (µg)	$MgSO_4$ (µg)	FeEDTA (µg)	$MnCl_2$ (µg)	H_3BO_3 (µg)	$ZnSO_4$ (µg)	$NaMoO_4$ (µg)	$CuSO_4$ (µg)	$Ca(H_2PO_4)_2$ - low P (µg)	$Ca(H_2PO_4)_2$ - normal P (µg)
0	600	RO water without nutrients										
4	250	RO water without nutrients										
8	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
11	250	RO water without nutrients										
14	250	RO water without nutrients										
18	250 (no P)	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	0	0
21	250	RO water without nutrients										
25	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
31	250	3792.2	5129.0	1505.0	55.1	6.3	17.0	8.1	0.1	3.2	73.2	1463.2
32	125	RO water without nutrients										
35	250	RO water without nutrients										
39	250	RO water without nutrients										

3.3. Results

3.3.1. *Shoot and root biomasses of IT98K-476-8 (tolerant), Aloka (susceptible), and progeny*

The shoot and root biomass results from the sand culture experiment for IT98K-476-8 and Aloka and their F_1, BC_1, and F_2 progeny are shown in Fig. 11. Also, shown are *t*-test comparisons of mean values. Fig. 12 shows images of the parents and their progeny before uprooting. Fig. 13 shows the distribution of shoot and root dry biomasses in the F_2, along with mean values for both parent varieties relative to the mean of the F_2.

The shoot dry biomass results show that F_1 and BC_1 to IT98K-476-8 plants have shoot dry biomasses statistically similar to the tolerant parent IT98K-476-8 indicating dominance gene action. The mean shoot dry biomass of F_1 plants was actually higher than for IT98K-476-8. Even though the F_2 shoot dry biomasses appeared as normally distributed, the mean of the F_2 shoot dry biomasses (1.41 g) was closer to the mean of IT98K-476-8 (1.57 g) than to Aloka (1.05 g). Also, the mean dry shoot mass of BC_1 plants to Aloka plants had a dry shoot biomass closer to Aloka indicating dominance. These shoot dry biomass results indicate low P tolerance is a heritable trait. Looking at shoot image results, it is evident that progeny inherit an increased stature and overall vigor from IT98K-476-8 but may also be inheriting an increase in branching number from Aloka that leads to their display of tolerance.

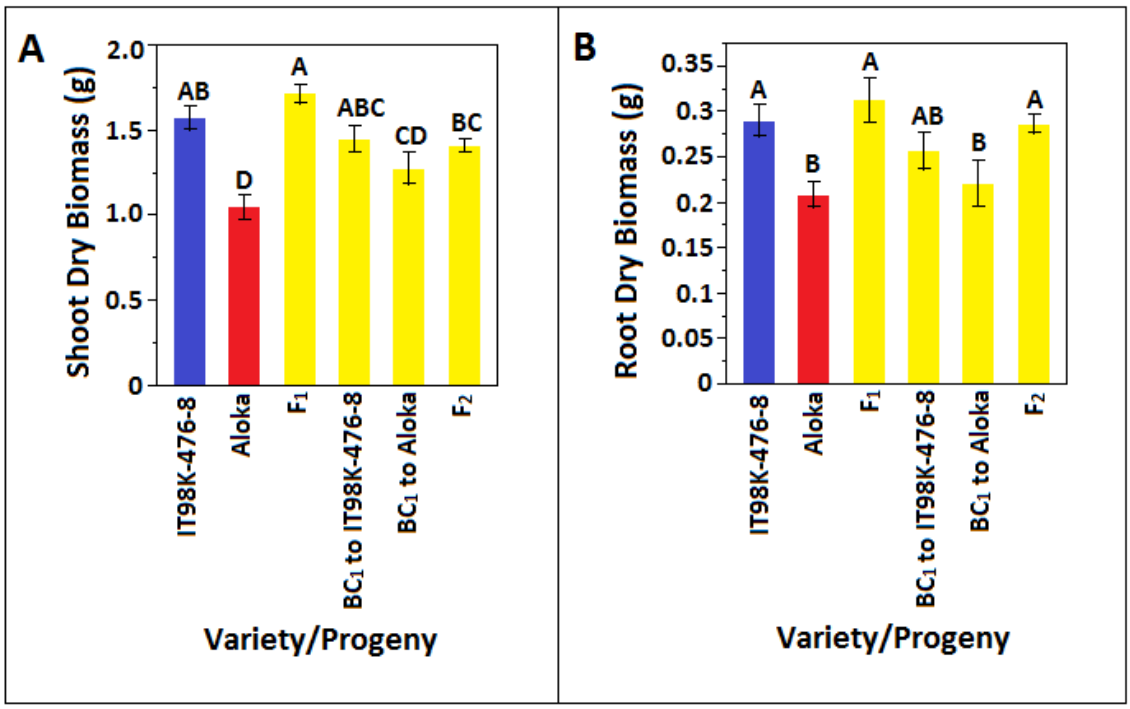

Fig. 11 Shoot (A) and root (B) dry biomass results for cowpea varieties IT98K-476-8 (tolerant) and Aloka (susceptible) and for the F_1, BC_1, and F_2 progeny of these two varieties, grown in sand culture with a low P treatment of 1.5 mg/kg P. The *t*-test comparisons of mean values ($p = 0.05$) are indicated in both graphs. Error bars represent standard error

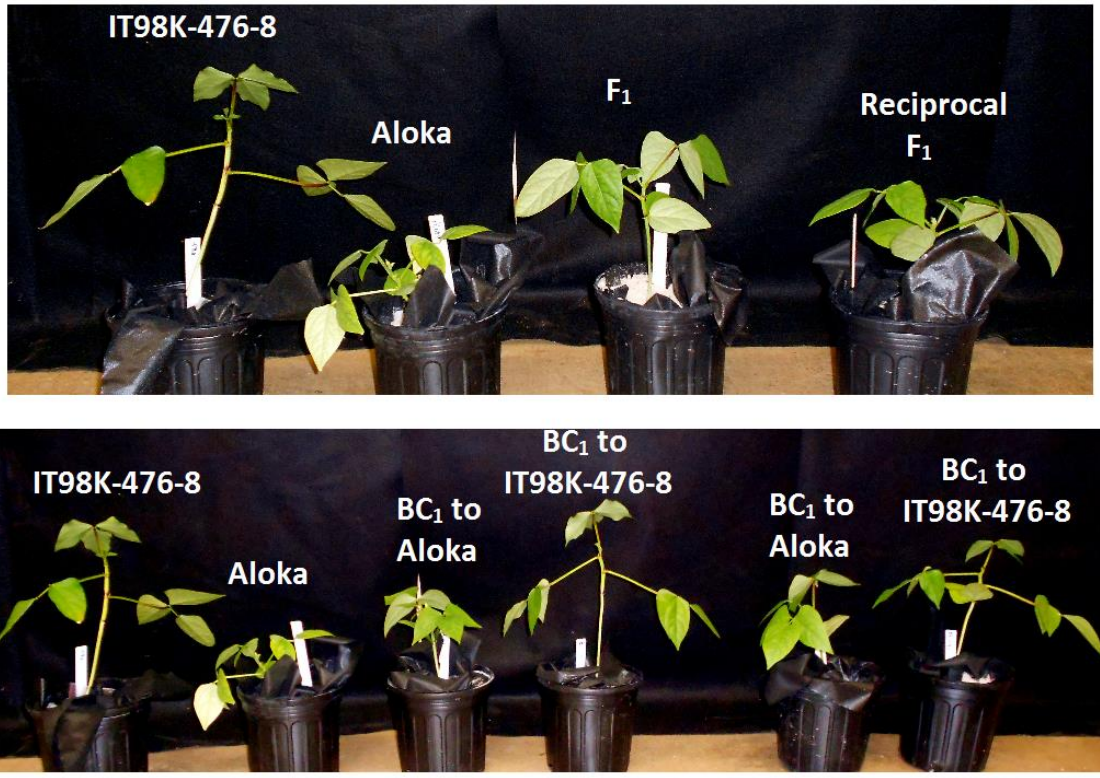

Fig. 12 Images of shoots before uprooting of IT98K-476-8, Aloka, and their progeny grown in a low P treatment of 1.5 mg/kg P. IT98K-476-8 is the tolerant parent to low P soils, and Aloka is the susceptible parent to low P soils. Results indicate that progeny adopt growth from IT98K-476-8 and perhaps even increased branching from Aloka that cause them to perform well in a low P treatment

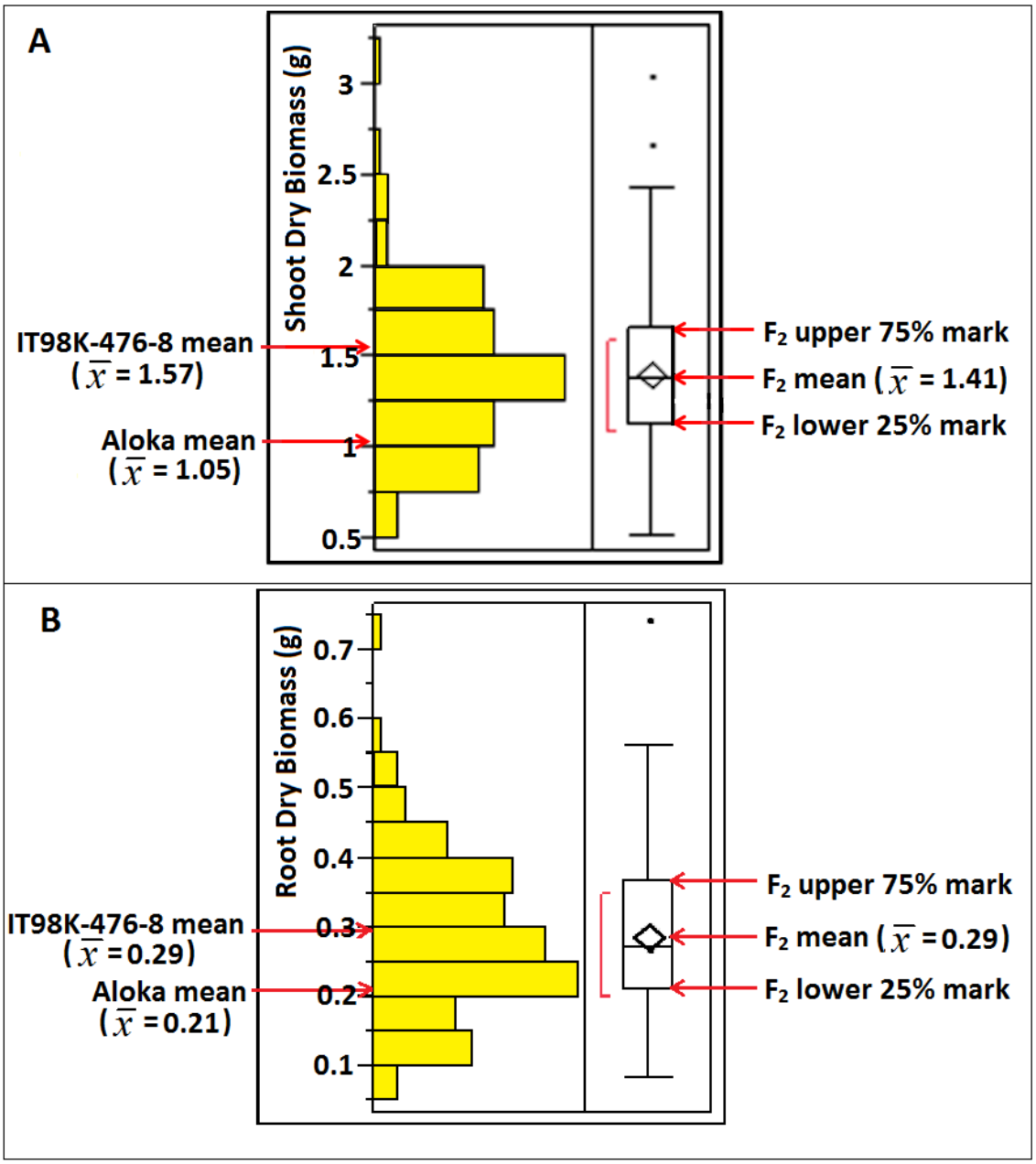

Fig. 13 The distribution of F_2 shoot (A) and root (B) dry biomasses in sand culture with a low P treatment of 1.5 mg/kg for IT98K-476-8 crossed to Aloka. The means of both varieties relative to the mean of the F_2 are indicated

The root dry biomass results show that both F_1 and F_2 plants have root dry biomasses statistically similar to the tolerant parent. The mean of the F_2 root dry biomasses (0.29 g) was the same as the mean of IT98K-476-8 to two significant figures. BC_1 to IT98K-476-8 plants had root dry biomasses intermediate to both parent varieties while BC_1 to Aloka plants had root dry biomasses more statistically similar to the susceptible parent Aloka. Root F_2 dry biomass values were similar in distribution to shoot dry biomass values, suggesting a possible proportional production in shoots and roots in response to minimal P availability aside from the sharper decrease in root biomass production in BC_1 results.

3.3.2. *Shoot and root biomasses of Big John (tolerant), Dan Ila (susceptible), and progeny*

The shoot and root biomass results from the sand culture experiment for Big John and Dan Ila and their F_1, BC_1, and F_2 progeny are shown in Fig. 14. Also, shown are *t*-test comparisons of mean values. Fig. 15 shows images of the parents and their progeny before uprooting. Fig. 16 shows the distribution of shoot and root dry biomasses in the F_2, along with mean values for both parent varieties relative to the mean of the F_2.

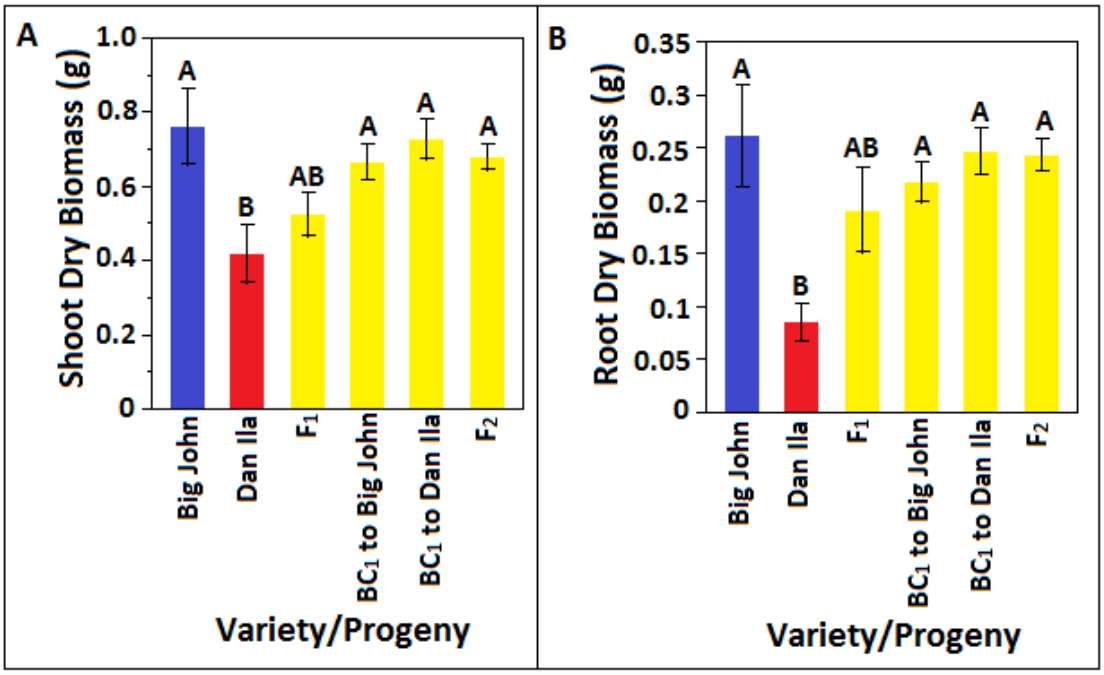

Fig. 14 Shoot (A) and root (B) dry biomass results for cowpea varieties Big John (tolerant) and Dan Ila (susceptible) and for the F_1, BC_1, and F_2 progeny of these two varieties, grown in sand culture with a low P treatment of 1.5 mg/kg P. The *t*-test comparisons of mean values ($p = 0.05$) are indicated in both graphs. Error bars represent standard error

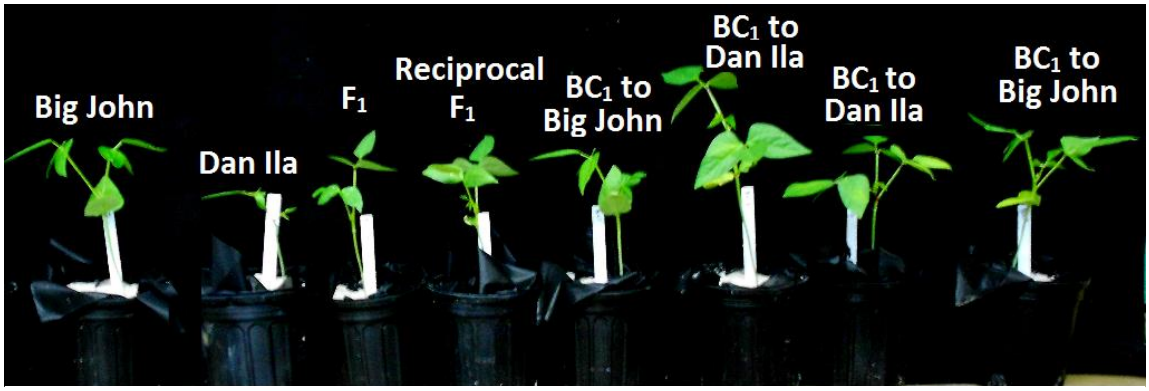

Fig. 15 Images of shoots before uprooting of Big John, Dan Ila, and their progeny grown in a low P treatment of 1.5 mg/kg P. Big John is the tolerant parent to low P soils, and Dan Ila is the susceptible parent to low P soils. Results indicate that progeny adopt growth from Big John that cause them to perform well in a low P treatment

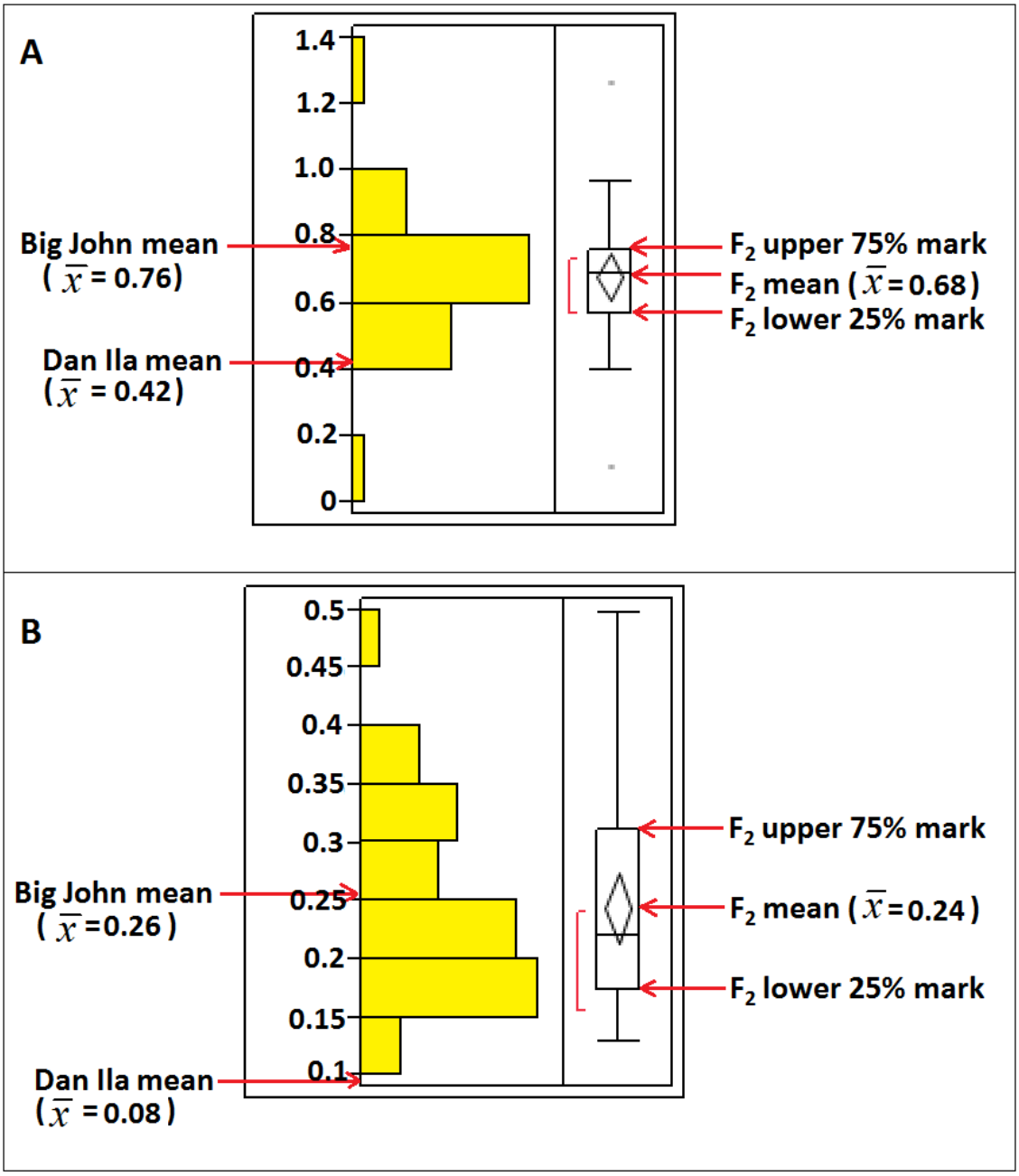

Fig. 16 The distribution of F_2 shoot (A) and root (B) dry biomasses in sand culture with a low P treatment of 1.5 mg/kg for Big John crossed to Dan Ila. The means of both varieties relative to the mean of the F_2 are indicated

The shoot dry biomass results show that BC_1 to Big John, BC_1 to Dan Ila, and F_2 plants have shoot dry biomasses statistically similar to the tolerant parent Big John. The mean shoot dry biomass of F_1 plants was lower than for Big John but higher than for Dan Ila. These F_1 results are lower than expected and may have been subject to experimental error, since in the next F_1 progeny study to be discussed, the F_1 consistently performed similar to or outperformed the tolerant parent. F_2 shoot dry biomasses were normally distributed and had a mean between both parents. The mean of the F_2 shoot dry biomasses (0.68 g) was significantly closer to the mean of Big John (0.76 g) than to Dan Ila (0.42 g). These shoot dry biomass results, like the results for IT98K-476-8 and Aloka, indicate that low P tolerance appears to be a heritable trait. BC_1 to Dan Ila plants performed closer to Big John, which was increased support for the high heritability of low P tolerance trait that had not been noted in the intermediate performance of BC_1 to Aloka plants of the previous screen.

The root dry biomass results show that BC_1 to Big John, BC_1 to Dan Ila, and F_2 plants have root dry biomasses statistically similar to the tolerant parent. F_1 plants had root dry biomasses intermediate to both parents. The mean of the F_2 root dry biomasses (0.24 g) was significantly closer to the mean of Big John (0.26 g). Like the previous study with IT98K-476-8 and Aloka, most of the root dry biomass values were similar in distribution to shoot dry biomass values, the results again suggest a proportional production in shoots and roots in response to minimal P availability.

3.3.3. *Shoot and root biomasses of other F_1 progeny*

The shoot and root biomass results and the respective shoot images from the sand culture experiment with low P and normal P treatments for other cowpea F_1 progeny crosses are shown in Fig. 17-23. Also shown are *t*-test comparisons of mean values (Fig. 17, 19, 21). Fig. 17 shows low P treatment results for F_1 progeny from crosses of tolerant to susceptible varieties, and Fig. 18 shows the shoot images of these F_1 progeny in both low P and normal P treatments. Fig. 19 shows low P treatment results for F_1 progeny from crosses of tolerant to partially tolerant varieties from high seed P, and Fig. 20 shows the shoot images of these F_1 progeny in both low P and normal P treatments. Fig. 21 shows low P treatment results for F_1 progeny from crosses of tolerant to other tolerant varieties, and Fig. 22 shows the shoot images of these F_1 progeny in both low P and normal P treatments. Fig. 23 shows the normal P treatment results for all F_1 progeny in the sand culture experiment.

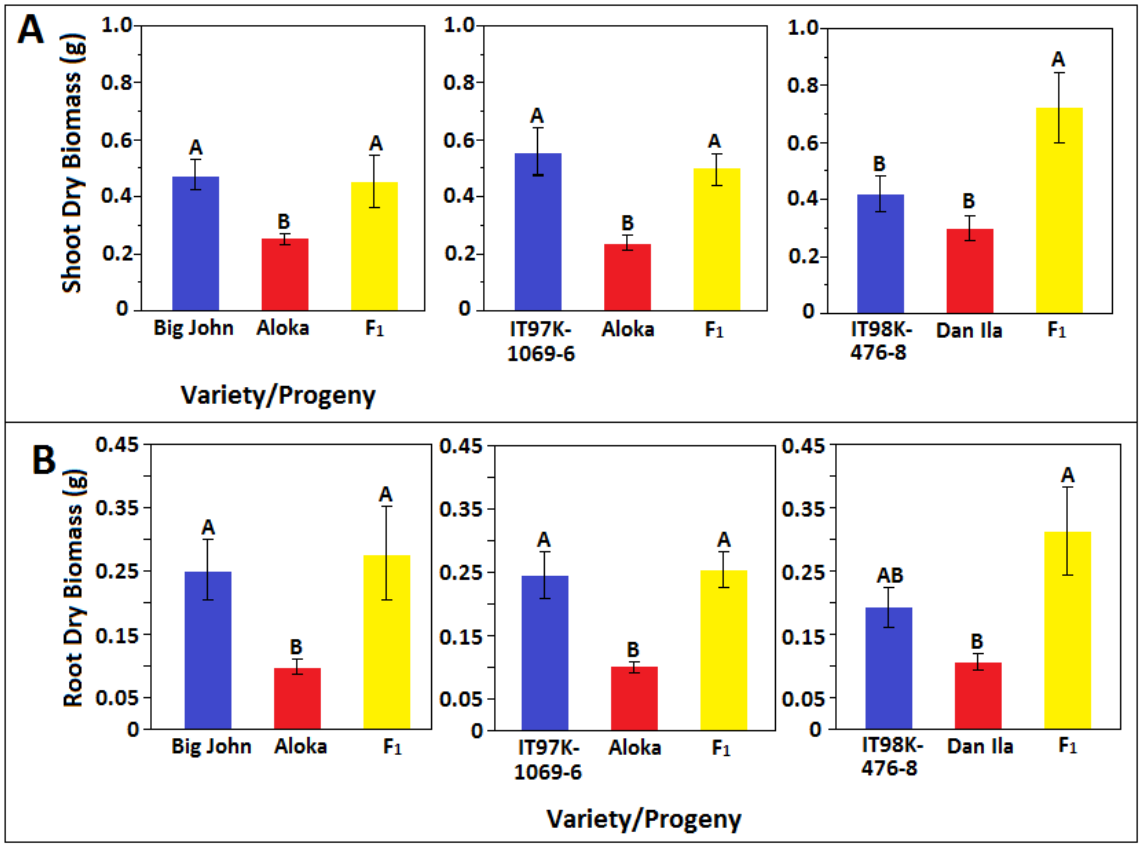

Fig. 17 Shoot (A) and root (B) dry biomass results in sand culture with a low P treatment of 1.5 mg/kg P for F_1 progeny of tolerant cowpea varieties crossed to susceptible varieties. Tolerant varieties are indicated in blue (left bar of graph), and susceptible varieties are indicated in red (center bar of graph). F_1 progeny are indicated in yellow (right bar of graph). The *t*-test comparisons of mean values (p = 0.05) are indicated in all graphs. Error bars represent standard error

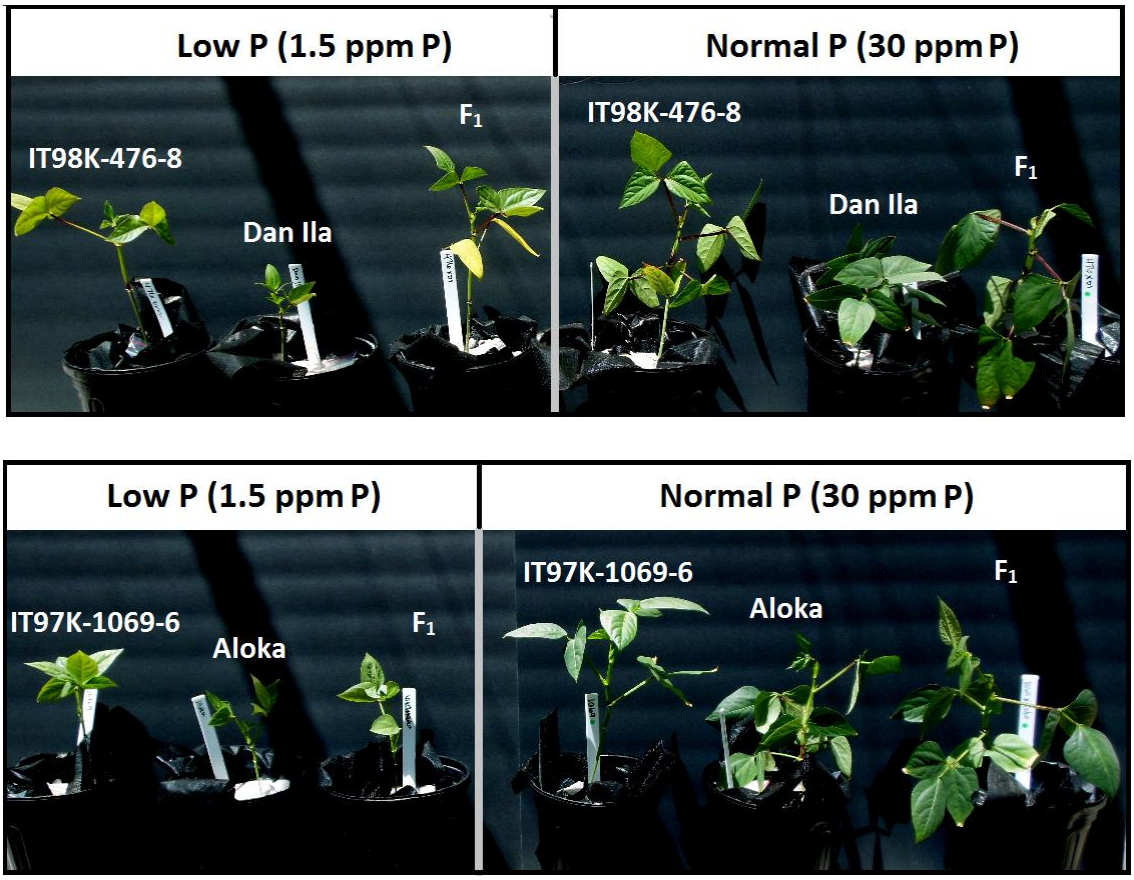

Fig. 18 Shoot images of the parents and F_1 progeny of crosses of tolerant to susceptible varieties grown in sand culture. Both low P (1.5 mg/kg P) and normal P (30 mg/kg P) treatments are shown. IT98K-476-8 and IT97K-1069-6 are the tolerant parents to P-deficiency, and Dan Ila and Aloka are the susceptible parents to P-deficiency. Results indicate that the F_1 perform more similarly to and can outperform the tolerant parent in a low P treatment, but the same is true in a normal P treatment

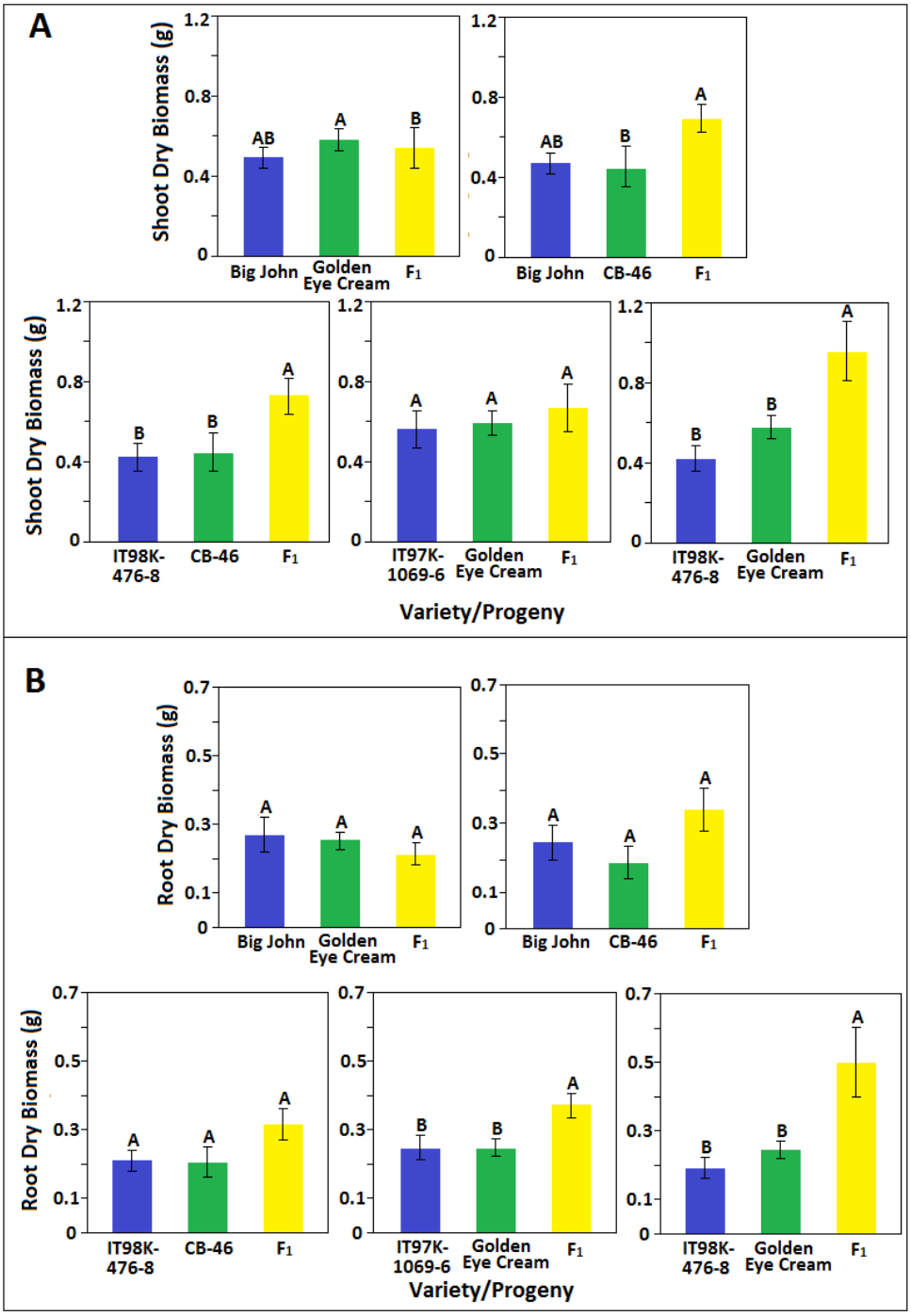

Fig. 19 Shoot (A) and root (B) dry biomass results in sand culture with a low P treatment of 1.5 mg/kg P for F_1 progeny of tolerant cowpea varieties crossed to partially tolerant varieties from seed P. Tolerant varieties are indicated in blue (left bar of graph), and partially tolerant varieties are indicated in green (center bar of graph). F_1 progeny are indicated in yellow (right bar of graph). The *t*-test comparisons of mean values (p = 0.05) are indicated in both graphs. Error bars represent standard error

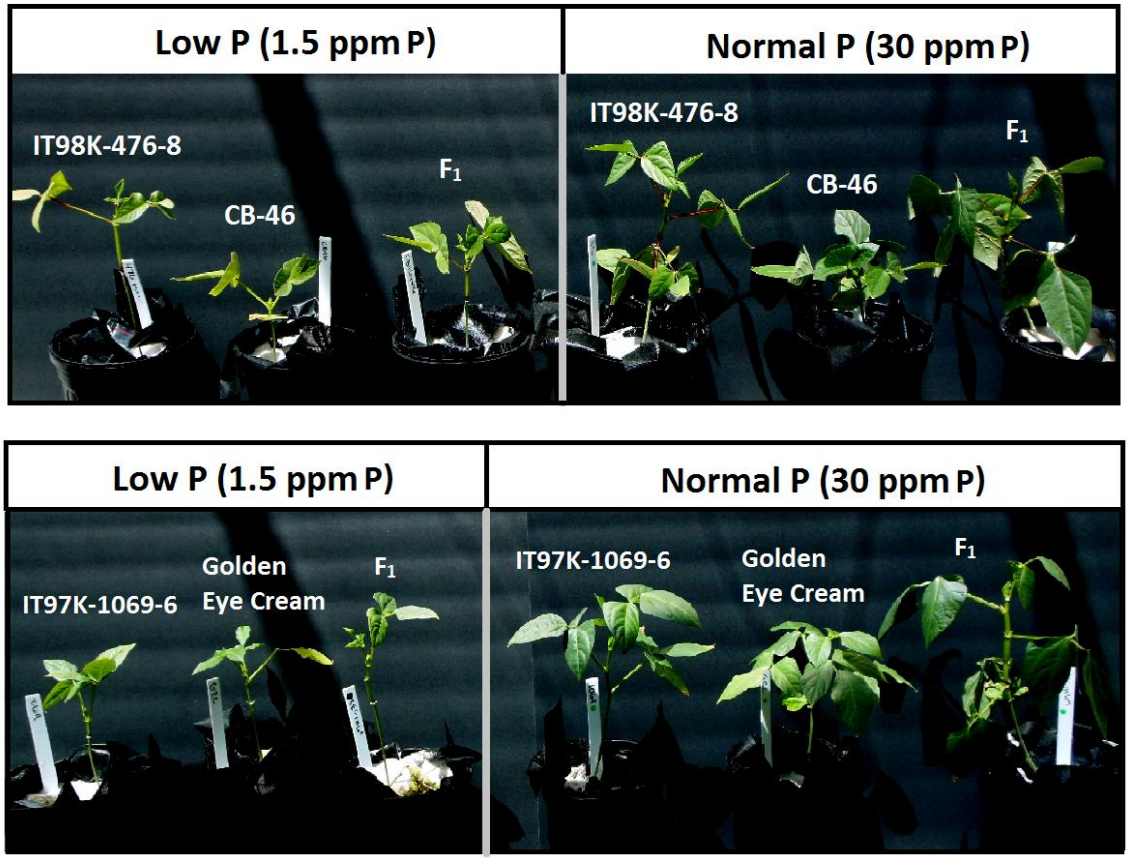

Fig. 20 Shoot images of the parents and F_1 progeny of crosses of tolerant to partially tolerant varieties grown in sand culture. Both low P (1.5 mg/kg P) and normal P (30 mg/kg P) treatments are shown. IT98K-476-8 and IT97K-1069-6 are the tolerant parents to P-deficiency, and CB-46 and Golden Eye Cream are the partially tolerant parents to P-deficiency. Results indicate that the F_1 perform more similarly to and can outperform the tolerant parent in a low P treatment, but the same is true in a normal P treatment

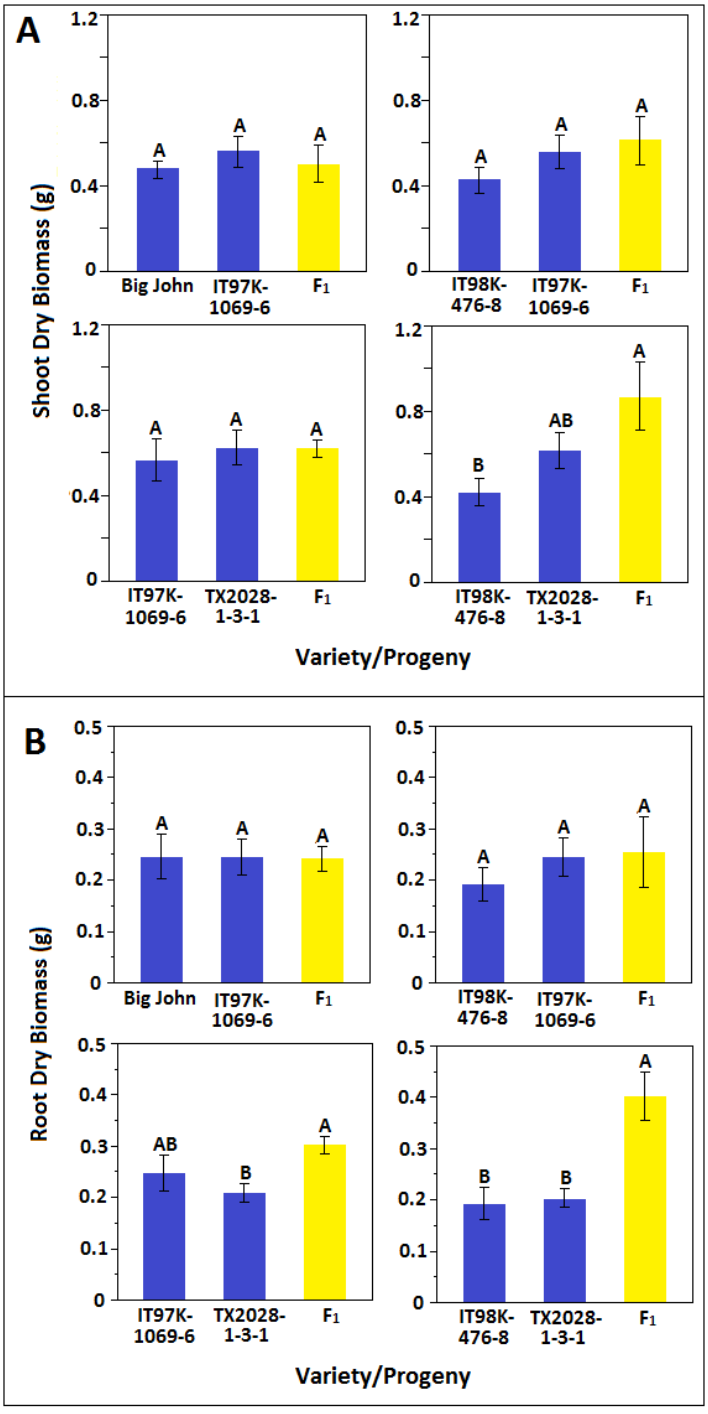

Fig. 21 Shoot (A) and root (B) dry biomass results in sand culture with a low P treatment of 1.5 mg/kg P for F_1 progeny of tolerant cowpea varieties crossed to other tolerant varieties. Tolerant varieties are indicated in blue (left and center bars of graph), and F_1 progeny are indicated in yellow (right bar of graph). The *t*-test comparisons of mean values (p = 0.05) are indicated in both graphs. Error bars represent standard error

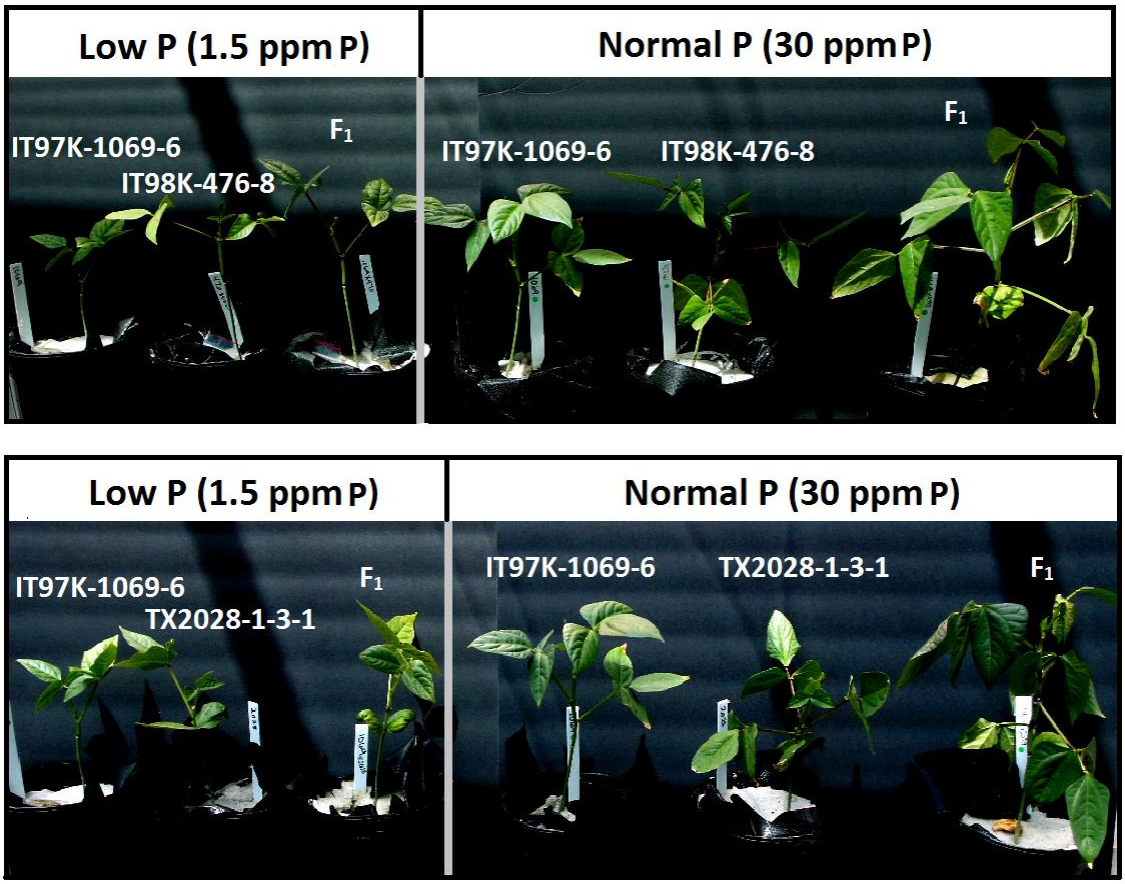

Fig. 22 Shoot images of the parents and F_1 progeny of crosses of tolerant to other tolerant varieties grown in sand culture. Both low P (1.5 mg/kg P) and normal P (30 mg/kg P) treatments are shown. All parents are tolerant parents to P-deficiency. Results indicate that the F_1 outperform both tolerant parent in a low P treatment, but the same is true in a normal P treatment

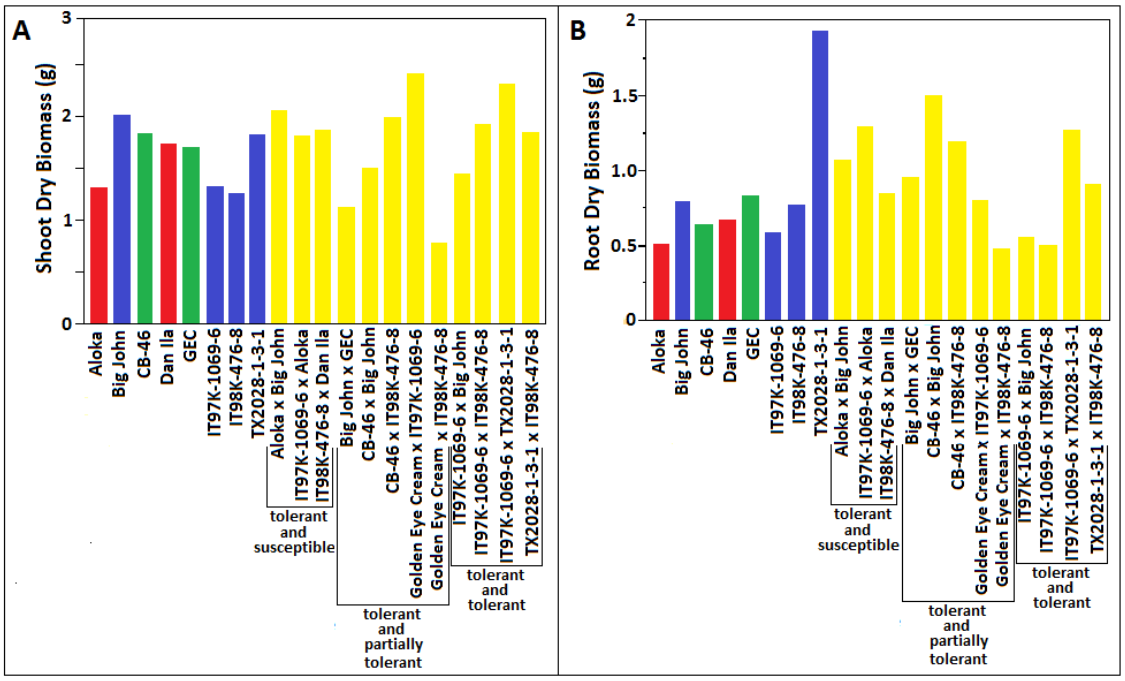

Fig. 23 Shoot (A) and root (B) dry biomass results in sand culture with a control normal P treatment of 30 mg/kg P for all F_1 progeny crosses and the parents. Tolerant varieties are indicated in blue; partially tolerant varieties are indicated in green; susceptible varieties are indicated in red; and F_1 progeny are indicated in yellow. Also indicated within each graph is the type of cross for each F1, whether by a tolerant, partially tolerant, or susceptible parents to P-deficiency

Fig. 17 results indicate that F_1 progeny from tolerant to susceptible crosses of cowpea varieties had dry shoot biomass results that were statistically similar or had a mean closer to that of the tolerant variety. For one of them, IT98K-476-8 crossed to Dan Ila, F_1 progeny outperformed both parents, as noted in the previous two studies of IT98K-476-8 crossed to Aloka. The root dry biomass results largely paralleled the shoot dry biomass results.

Fig. 19 results indicate that F_1 progeny from tolerant to partially tolerant crosses of cowpea varieties had dry shoot biomass results that were significantly higher than both parents or were statistically similar to one or both parents. For IT98K-476-8 crossed to CB-46 and IT98K-476-8 crossed to Golden Eye Cream, the F_1 had a significantly higher shoot biomass. For Big John crossed to CB-46, the F_1 had a significantly higher biomass than CB-46. For Big John crossed to Golden Eye Cream, the F_1 had a shoot biomass statistically similar biomass to Big John but not Golden Eye Cream. For IT97K-1069-6 crossed to Golden Eye Cream, the F_1 had a shoot biomass statistically similar to both parents. The root dry biomass results paralleled the shoot dry biomass results, though the statistical mean comparison *t*-test values did start to deviate from the shoot dry biomass results in most of the graphs. The deviations can be noted in Fig. 19.

Fig. 21 results indicate that F_1 progeny from tolerant to tolerant crosses of cowpea varieties had dry shoot biomass results that were statistically similar to the parents. For one of them, IT98K-476-8 crossed to TX2028-1-3-1, F_1 progeny significantly outperformed both parents. The root dry biomass results paralleled the shoot dry biomass results, though the statistical mean comparison *t*-test values did start to deviate from the shoot dry biomass results in a couple graphs. The deviations can be noted in Fig. 21.

Fig. 23 results indicate a lack of significant differences in shoot and root dry biomasses for all varieties and F_1 progeny when grown in a normal P treatment. The exception is significantly high root growth in TX2028-1-3-1 in the normal P treatment.

These results support that variation in shoot and root dry biomasses under the low P treatment are actual responses to the P treatment and not from natural variation among varieties in shoot or root growth rate or pattern.

3.3.4. *Genetic effects and heritability estimates of low P tolerance in "IT98K-476-8 to Aloka" and "Big John to Dan Ila" crosses*

Estimates of genetic effects for low P tolerance from shoot biomass results for each cross and their progeny are given in Table 15. Results indicate a significant positive additive effect for low P tolerance in both crosses. For Big John crossed to Dan Ila, a significant positive additive x dominance effect was identified. Results did not identify significant dominance, additive x additive, or dominance x dominance effects for either cross.

Variance and heritability estimates for both crosses are indicated in Table 16. Results indicate a high narrow-sense heritability of roughly 0.74 for both crosses, which in addition to the significant additive effects, indicate that low P tolerance is a heritable additive trait. Such results indicate favorable potential breeding of the low P tolerance trait.

Table 15 Estimates and significance levels for mean (m), additive (a), dominance (d), additive x additive (aa), additive x dominance (ad), and dominance x dominance (dd) effects for low P tolerance from shoot biomass results of two crosses, IT98K-476-8 to Aloka and Big John to Dan Ila

Parameter	Estimate	Significance level
	IT98K-476-8 x Aloka	
m	1.495	<0.0001
a	0.263	<0.0001
d	-0.056	0.532
aa	-0.183	0.551
ad	-0.183	0.543
dd	0.779	0.205
	Big John x Dan Ila	
m	0.515	0.024
a	0.171	0.01
d	0.65	0.291
aa	0.076	0.722
ad	-0.466	0.032
dd	-0.64	0.15

Table 16 Estimates for environmental variance (V_E), additive variance (V_A), dominance variance (V_D), broad-sense heritability (h^2_B), and narrow-sense heritability (h^2_N) for low P tolerance from shoot biomass results of two crosses, IT98K-476-8 to Aloka and Big John to Dan Ila

Cross	V_E	V_A	V_D	h^2_B	h^2_N
IT98K-476-8 x Aloka	0.078	0.129	-0.034	0.548	0.748
Big John x Dan Ila	0.029	0.029	-0.019	0.26	0.742

Estimates of the number of genes involved were 0.268 for IT98K-476-8 to Aloka and 0.503 for Big John to Dan Ila. These results approximate one gene or locus as responsible for low P tolerance in both crosses. Together, all results for genetic effects, heritability, and gene number seem to indicate that a highly heritable additive gene is responsible for low P tolerance.

The same calculations for genetic effects and heritability were applied to root biomass results and are presented in Tables 17 to 18. Results indicate a significant positive additive effect for both crosses. For IT98K-476-8 to Aloka, a negative significant effect for dominance, negative significant effect for additive x additive, and positive significant effect for dominance x dominance were identified. For Big John crossed to Dan Ila, like for shoot biomass results, a significant positive additive x dominance effect was identified.

Table 17 Estimates and significance levels for mean (m), additive (a), dominance (d), additive x additive (aa), additive x dominance (ad), and dominance x dominance (dd) effects for root biomass results of two crosses, IT98K-476-8 to Aloka and Big John to Dan Ila

Parameter	Estimate	Significance level
	IT98K-476-8 x Aloka	
m	0.440	<0.0001
a	0.041	0.0144
d	-0.485	0.0488
aa	-0.190	0.0247
ad	-0.009	0.9145
dd	0.358	0.0345
	Big John x Dan Ila	
m	0.220	0.0233
a	0.088	0.0021
d	0.126	0.6288
aa	-0.046	0.6115
ad	-0.233	0.0123
dd	-0.154	0.4106

Heritability results indicate a high narrow-sense heritability of roughly 0.790 for IT98K-476-8 to Aloka and 0.936 for Big John to Dan Ila for both crosses, which supported by significant additive effects, indicate that root biomass production is a highly heritable additive trait. However, results for IT98K-476-8 to Aloka also suggested a significant dominance or dominance x dominance effect, which were backed by a broad-sense heritability of 0.588. The broad-sense heritability estimate of 0.588 is relatively high, especially when compared to the same estimate of 0.300 for Big John to Dan Ila, to suggest that there is a root growth mechanism involved in the cross of IT98K-476-8 to Aloka that may not be involved in that of Big John to Dan Ila.

Table 18 Estimates for environmental variance (V_E), additive variance (V_A), dominance variance (V_D), broad-sense heritability (h^2_B), and narrow-sense heritability (h^2_N) for root biomass results of two crosses, IT98K-476-8 to Aloka and Big John to Dan Ila

Cross	V_E	V_A	V_D	h^2_B	h^2_N
IT98K-476-8 x Aloka	0.005	0.010	-0.003	0.588	0.790
Big John x Dan Ila	0.005	0.007	-0.005	0.300	0.936

Estimates of the number of genes involved were 0.0831 for IT98K-476-8 to Aloka and 0.552 for Big John to Dan Ila. The results for Big John to Dan Ila indicate approximately one gene or locus is involved with root biomass production. The results for IT98K-476-8 to Aloka indicate that the differences in root biomass production between IT98K-476-8 and Aloka were too small to detect the number of genes involved, leading to an estimate close to 0.

3.4. Discussion

Results from these studies overall indicate low P tolerance is a highly heritable trait that can readily be bred. The main cross of interest was IT98K-476-8 to Aloka which has the highest number of individuals, but another cross of Big John to Dan Ila with fewer individuals was included as a check of results. Also, F_1 individuals of several other crosses were tested in a separate study to further test the inheritance of low P tolerance.

Most of the progeny from the shoot biomass results for IT98K-476-8 to Aloka and Big John to Dan Ila had a mean closer to the tolerant than susceptible parent with the

exception of the BC_1 to Aloka and the F_1 for Big John to Dan Ila. Additional phenotyping of F_1 individuals from tolerant to susceptible crosses showed that F_1 seed perform more similar to the tolerant parent or even outperform the tolerant parent, suggesting hybrid vigor and heterosis to be present. The F_1 seed tested in the cross of Big John to Dan Ila may have underperformed from an unknown cause.

Shoot biomass results for IT98K-476-8 to Aloka and Big John to Dan Ila indicated significant positive additive effects and high narrow-sense heritability for low P tolerance in cowpea. For Big John to Dan Ila, there was a possible negative additive x dominance effect, but broad-sense heritability estimates for Big John to Dan Ila were low. These results deviate from those for PAE in cowpea by Ojo *et al*. (2007) and in maize by Parentoni *et al*. (2010). Instead, these results agreed with those of Da Silva *et al*. (1992) and Araújo *et al*. (2005) which suggest additive over dominance effects as important for crop tolerance to P-deficiency. Also, the significant narrow-sense heritability identified in these results is one of the first reported since other studies had focused on and identified significant broad-sense heritability. Gene number calculations identified only one gene or locus as responsible for low P tolerance in cowpea. While many physiological and biochemical traits can contribute to low P tolerance in a variety, there does exist the possibility of a downstream or strong effect trait that is largely responsible for tolerance.

Studies on the genetic effects and heritability of root biomass production for both crosses indicated positive additive gene effects and significant narrow-sense heritability. However, the results for IT98K-476-8 and Aloka, also indicated possible significant

negative dominance, negative additive x additive, and positive additive x dominance gene effects, in addition to a significant broad-sense heritability estimate. These results show that there are possible dominance, additive x additive, and dominance x dominance gene effects for root biomass production present in the IT98K-476-8 to Aloka cross that are either not present in the Big John to Dan Ila cross or were not detected because of a smaller population size in the study of the Big John to Dan Ila cross. Since root biomass production is not a traditional measure of low P tolerance and is likely not the direct or only cause for low P tolerance, a change in genetic effect and heritability estimates from root biomass results relative to shoot biomass results is acceptable and expected. It was found though, like for shoot biomass results, only one gene appeared to be responsible for root biomass production, perhaps because of a possible downstream or strong effect trait controlling root production.

The high broad-sense heritability estimate for root biomass production in our study of IT98K-476-8 to Aloka supports the same estimate of Araújo *et al*. (2005) for the heritability of root biomass production in a P-deficient soil, but their study did not identify significant dominance effects for root biomass production. Overall, the possibility of a dominance effect for root biomass production in P-deficient soil is possible, but significant additive effects are also involved.

Results of the other F_1 crosses showed that, whether the cross is a tolerant to susceptible, a tolerant to semi-tolerant, or a tolerant to tolerant, the F_1 progeny perform more similarly to the tolerant parent(s) and even outperform the tolerant parent(s) in some crosses. Such results indicate a possible heterotic effect for the low P tolerance

trait. Such heterotic effects in the F_1 had also been observed by Ojo *et al*. 2007 for increased yield and seed P when growing cowpea in P-deficient soils supplemented with RP.

Since additive effects over dominance effects are more favored by breeders because they can be fixed, our results indicate that low P tolerance is a trait that can be readily bred for in cowpea. Also, the responsible additive effect physiological or genetic mechanisms leading to tolerance should be readily identifiable. Such knowledge will pave the way for breeding cowpea and other crop species to do well in P-deficient soils as P becomes an increasingly limited nutrient in the future.

4. QTL MAPPING OF LOW P TOLERANCE IN COWPEA

4.1. Introduction

Mapping for crop tolerance to P-deficient soils is on the rise as soils in many developing countries, particularly in Africa, have poor soil fertility and inaccessibility to fertilizer. The spread of soil P-deficiency is of growing concern since many predictions exist that P-reserves worldwide will be largely depleted within the next century. Recent well-known studies for mapping tolerance to P-deficient soil conditions have been conducted in Asian rice varieties, and the *Pup1* gene locus was identified (Wissuwa *et al*. 2002). Within the *Pup1* gene locus, the protein kinase PSTOL1 was associated with tolerance (Gamuyao *et al*. 2012), and an increase in root growth was shown as one of the key physiological effects from *Pup1* that leads to P-deficiency tolerance (Wissuwa *et al*. 2002). The *Pup1* gene has since been introgressed into rice varieties for growth in P-deficient soils across Asia. Recently, studies have also started for developing P-deficiency tolerant rice varieties for Africa as well. Studies conducted by the Japan International Research Center for Agricultural Sciences (JIRCAS) and the Africa Rice Center (AfricaRice) have utilized information from research on the *Pup1* gene to identify an African rice variety CG14 that has a variation of the *Pup1* allele that differs by just 35 nucleotides but also confers tolerance to P-deficient soils (Yanagihara *et al*. 2010).

Cowpea, as the staple legume across Africa, is a vital economic crop for food security. Much African soil, particularly in West Africa, is plagued by deficiencies in P

that are not readily supplemented because of the high importation and production costs for fertilizer in Africa. Thus, West African farmers cannot afford fertilizer and are left to try to maximize their crop production with soils that are severely handicapped for potential yield. Maximizing cowpea yield independent of soil P-deficiency in West Africa would be a large economic and food security boost for farmers.

Cowpea varieties have been identified for tolerance to P-deficient conditions (see Section 2). One of these varieties IT98K-476-8 has been crossed to a susceptible variety Aloka to create an F_6 Recombinant Inbred Line (RIL) population for mapping low P tolerance. In this study, this population was used for mapping low P tolerance with SSR markers and in the future will be used for developing SNP markers for further fine-mapping of low P tolerance.

In past studies, molecular markers have been utilized in cowpea to study genetic diversity, develop linkage maps, and map a select few traits of interest: thrips resistance (Omo-Ikerodah *et al*. 2008; Muchero *et al*. 2010a), drought resistance (Muchero *et al*. 2009b and 2010b), bacterial blight resistance (Agbicodo *et al*. 2010), and *Macrophomina phaseolina* resistance (Muchero *et al*. 2011). Past markers that have been used include allozymes, RAPDs, AFLPs, RLFPs, SSRs, and SNPs. This study utilizes genome-derived SSR markers from the Cowpea Genomics Initiative (CGI) at the University of Virginia to map low P tolerance in cowpea. This study lays the foundation for identification of any linkage groups or QTL in cowpea associated with low P tolerance. Also, this study can lead to a possible identification of a heterologous gene locus to *Pup1* in cowpea.

It is suspected that any QTL identified will likely also be associated with secondary physiological responses to soil P-deficits such as enhanced use of stored P in translocation between plant tissues, enhanced root growth, increased root surface anion exchange capacity, increased root hair formation, etc. Such associations have commonly been found not only in studies of *Pup1* in rice but also in studies of low P tolerance QTL in other crop species, such as common bean, soybean, rice, maize, and wheat (Collins *et al*. 2008). The cowpea variety, IT98K-476-8, used for mapping in this study has been linked to low P tolerance (see Section 2). Thus, the mapping conducted in this study will likely be associated with QTL for low P tolerance.

4.2. Materials and Methods

4.2.1. *Phenotyping by sand culture the RIL population*

An F_6 RIL population of 125 individuals for mapping low P tolerance in cowpea was developed by single seed descent (SSD) from a cross of a variety positive for low P tolerance, IT98K-476-8, to a variety negative for low P tolerance, Aloka. Both are Nigerian varieties that flower at medium to late maturity.

Texas A&M University greenhouse facilities were used to grow cowpea varieties in a controlled environment. Greenhouse conditions were at 27 ºC daytime temperature and 23 ºC nighttime temperature. A daily photoperiod of 14 hours was applied by supplementing natural light with artificial lights if natural light reached below 700 W/m^2. Five-hundred one-gallon pots (cylindrical shape, 14.5 cm diameter by 16.5 cm height) were prepared for screening under either low P (1.5 mg/kg P) or normal P (30

mg/kg P) treatments. Pots were lined with landscaping material cut into 18 in x 18 in (45.72 cm x 45.72 cm) pieces. Lined pots were filled with “Kosse White” silica sand (U.S. Silica, Kosse, TX)), which ranges in particle size from roughly 0.27 to 0.95 mm. Lining the pots ensured sand would not escape through pot drain holes, but nutrient solution could readily flow through. For each RIL individual, four pots were planted with two seeds per pot and later thinned to one seedling per pot at 10 DAP. Seeds were planted with Royal Peat Legume Seed Inoculant (Becker Underwood, St. Joseph, MO). Three pots of each RIL individual were screened under the low P treatment, and one pot of each RIL individual was screened under the normal P treatment as a control.

Nutrient solutions were modified from Johnson *et al.* (1994) and created with reverse osmosis (RO) water. The nutrient solution composition is shown in Table 8. The normal P treatment contained 0.5 mM $Ca(H_2PO_4)_2$ instead of 25.0 µM $Ca(H_2PO_4)_2$ for the low mg/kg P treatment. Pots were watered with nutrient solution and RO water in a modified version of the sand culture screen described in Section 2. Pots were watered with nutrient solutions accordingly: 300 mL at 9 DAP, 200 mL at 3 DAP, and 300 mL at 30 DAP. Pots were watered with RO water accordingly: 500 mL at planting, 250 mL at 9 DAP, 250 mL at 16 DAP, 250 ml at 29 DAP. All plants were uprooted at 38 DAP, and the shoots were separated from roots at the crown for drying. Shoot samples were dried overnight at 75 ºC, and then shoot dry biomasses were measured.

Tolerance of RIL individuals was determined by a P susceptibility index (PSI). The PSI was calculated as

$$PSI = \frac{1 - [Y_1 / Y]}{1 - [X_1 / X]}$$

where: Y_1 is the average dry shoot biomass in the low P treatment for a RIL individual; Y is the average dry shoot biomass in the low P treatment for all RIL individuals; X_1 is the average dry shoot biomass in the normal P treatment for a RIL individual; and X is the average dry shoot biomass in the low P treatment for all RIL individuals. RIL individuals with a PSI lower than 1.1 were considered to have low P tolerance while individuals with a PSI of 1.1 or higher were considered to not have low P tolerance.

4.2.2. *Mapping for low P tolerance*

Three-hundred ninety-six genome-derived SSR markers from the Cowpea Genomics Initiative (CGI) at the University of Virginia were run on IT98K-476-8 and Aloka, and 75 polymorphic markers were identified. These polymorphic markers were run on 120 F_6 RIL individuals for mapping linkage groups and identifying QTL for low P tolerance.

Parent and RIL genomic DNA was extracted using the Plant DNA Extraction Protocol for DArT (http://www.diversityarrays.com/sites/default/files/pub/DArT_DNA_isolation.pdf) (Diversity Arrays Technology 2013). DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). DNA was diluted to working concentrations of 25 ng/µL with ddH_2O. A 1x PCR reaction mixtures for each primer and DNA strain were created with: 5x Green GoTaq® Flexi Buffer (Promega, Madison, WI), $MgCl_2$ (Promega, Madison, WI), dNTPS (Thermo Fisher Scientific, Waltham, MA), ddH_2O, 50% dimethyl sulfoxide (DMSO)

(Sigma-Aldrich, St. Louis, MO), Taq DNA Polymerase (New England BioLabs, Ipswich, MA), forward and reverse primers, and DNA. These reactions were run on thermocyclers with the following program: 94 ºC for 5 min of initial denaturation, 35 cycles of 94 ºC for 30 sec followed by 55 ºC for 30 sec followed by 72 ºC for 1 min, and 72 ºC for 10 min of final extension. PCR plates were stored at -20 ºC until analysis.

PCR plates were run on a *Fragment Analyzer*^{TM} Automated CE System (Advanced Analytical Technologies, Ames, IA) with dsDNA Reagent Kits, 35-500 bp (Advanced Analytical Technologies, Ames, IA) to identify polymorphic markers for the two parents. After running 396 genome-derived SSR markers, 75 markers were identified as polymorphic. PCR plates for these markers were then run on 120 individuals of the IT98K-476-8 to Aloka RIL population on the *Fragment Analyzer*^{TM} Automated CE System. Raw data from the system was analyzed with PROSize 2.0 software (Advanced Analytical Technologies, Ames, IA) to score polymorphic bands of the parents and RIL individuals.

A linkage map of all 75 markers was constructed with QTL IciIMapping (ICIM) (The Quantitative Genetics Group, Beijing, China). Previous linkage maps constructed (from Xu *et al*. 2011, Andargie *et al*. 2011, and the CGI) were used to create anchors in ICIM of known markers in the same linkage groups. The resultant linkage map was used to map QTL for low P tolerance in MapQTL® 6 (Kyazma, Wageningen, Netherlands). Multiple interval mapping (MIM) was used with parameters set at P=0.05 significance and 10,000 permutations. Mapping was conducted on the following phenotyping results: shoot dry biomass (g) in the low P treatment, PSI values, and a positive/negative

qualitative score for low P tolerance. The positive/negative qualitative score for low P tolerance was determined by comparing each RIL line's PSI value to the known PSI values of the parent varieties to categorize the RIL as positive or negative for low P tolerance.

4.3. Results

4.3.1. *Phenotyping of the RIL population*

The RIL shoot biomass results in the low P treatment and PSI values calculated from shoot biomass results in both the low and normal P treatment are shown in Fig. 24. Graphs show a normal distribution. The mean and median PSI values for the RIL population centered more closely to the PSI for IT98K-476-8 than to the PSI for Aloka confirming previous studies. PSI values were used to score a RIL line as tolerant or susceptible, and these scores were used in MapQTL® 6 as an index for mapping QTL for low P tolerance. Also, PSI values and shoot biomass in the low P treatment were used as indices for mapping QTL for low P tolerance.

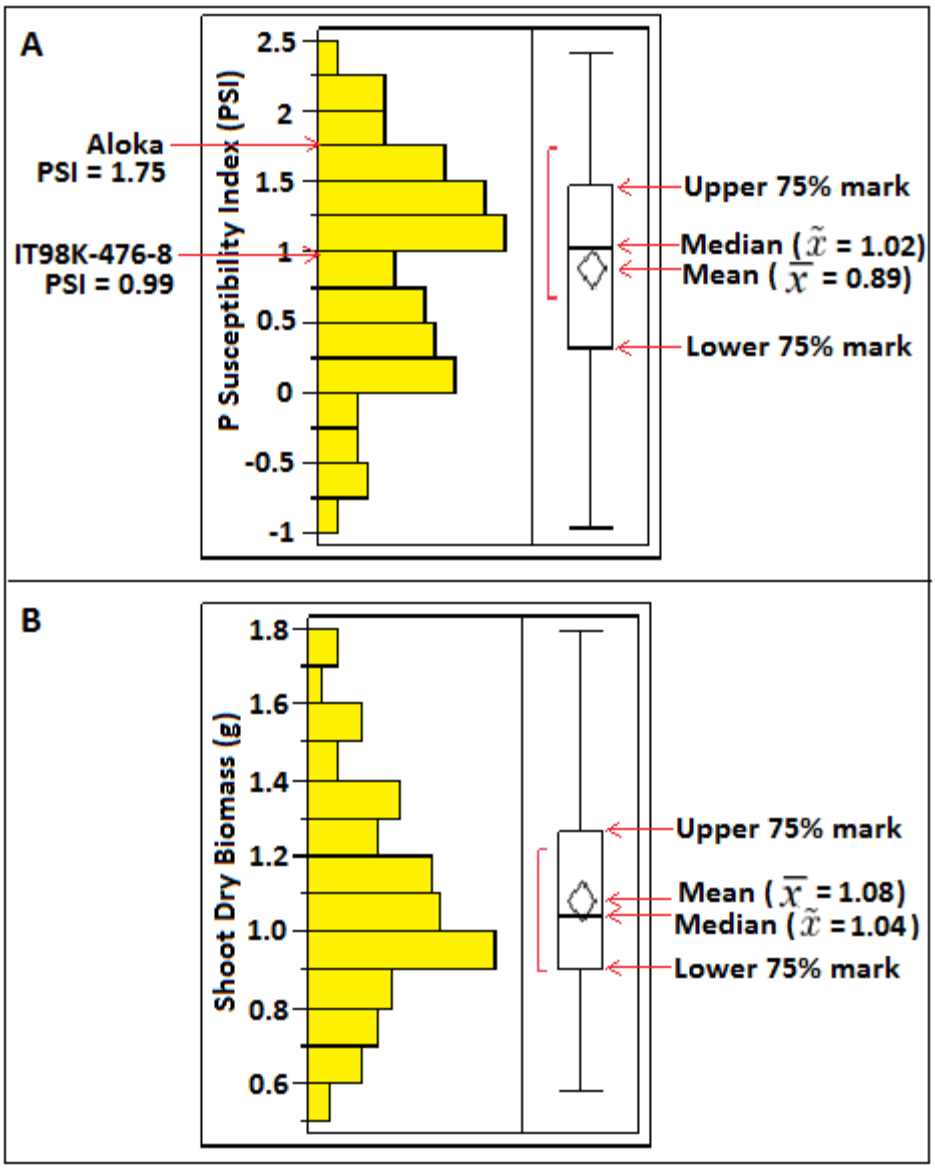

Fig. 24 Shoot biomass results for a RIL population of 120 individuals from a cross of IT98K-476-8, positive for low P tolerance, to Aloka, negative for low P tolerance. PSI results (A) show a distribution of PSI values encompassing the original PSI values of Aloka and IT98K-476-8. The mean and median PSI values center more closely to IT98K-476-8, positive for low P tolerance. Shoot biomass results in the low P treatment (B) show a normal distribution of RIL shoot biomasses from 0.5 to 1.8 g. Shoot biomass in the low and normal P treatments were used to calculate PSI values (A)

4.3.2. *Linkage group map*

Markers were assigned into 11 linkage groups spanning approximately 868 cM shown in Fig. 25. Only two markers, RB12 and MA118, could not be assigned into linkage groups and were placed together in the last group of Fig. 25 but are not linked. Two linkage groups shown, LG3A and LG3B, can be combined into the same linkage group, but the linkage group was arbitrarily split into two halves since LG3A has the QTL of interest but not LG3B. The two groups may come from different regions of the same chromosome. The length of the linkage groups ranges from 11.24 cM (LG5) to 125.24 cM (LG3A), and the mean was 78.92 cM. The number of markers per LG ranged from 3 (LG5) to 9 (LG3A and LG6), and the mean was 6.64. These calculations are taking into account linkage groups LG3A and LG3B as two separate groups and not including LG11 since its markers are not linked.

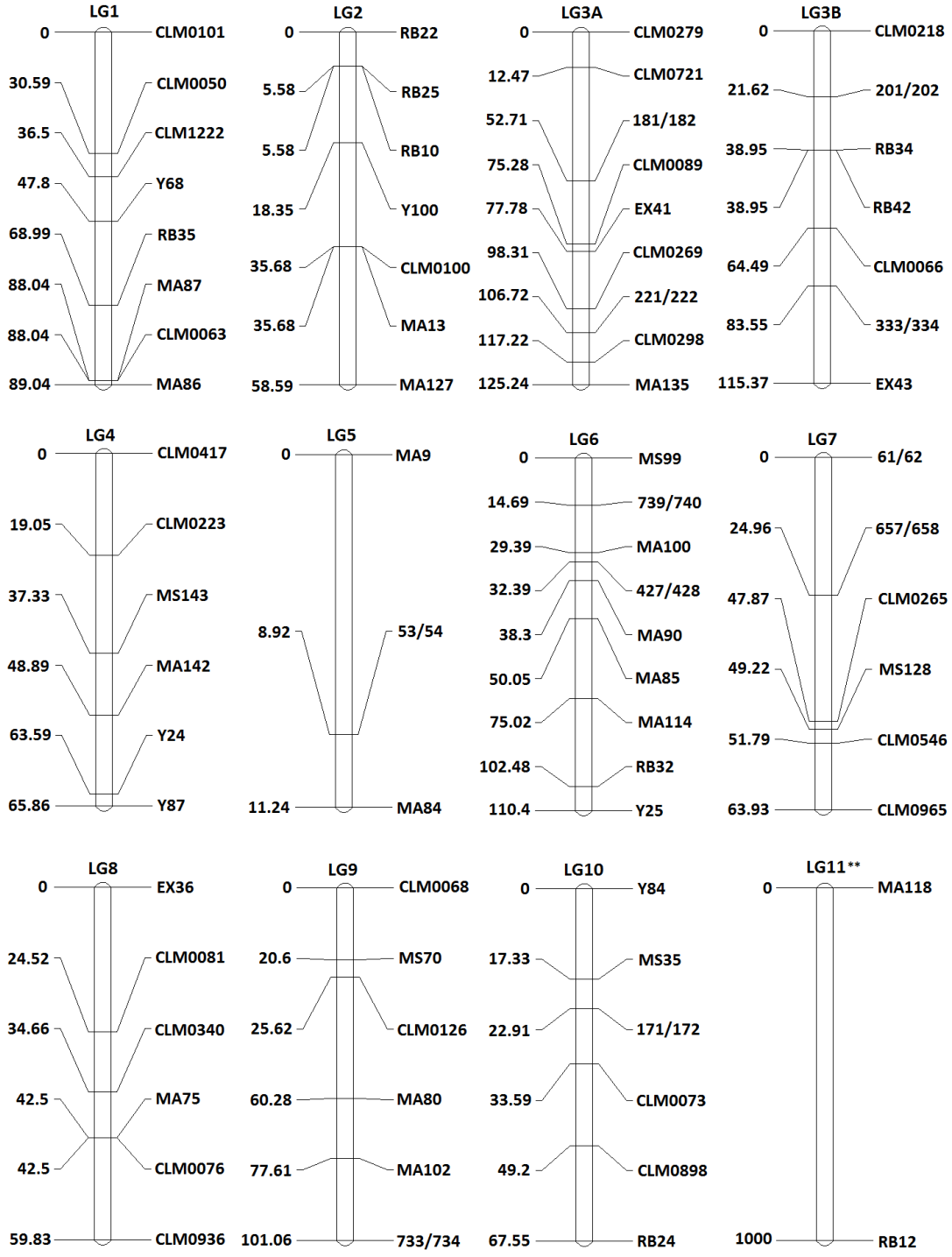

Fig. 25 Linkage groups mapped from marker analysis of 120 RIL individuals to the F_6 generation of the cross of IT98K-476-8 to Aloka. Previous linkage maps for many of these markers from Xu *et al*. (2011), Andargie *et al*. (2011), and the CGI, were used to assign anchor groups.**LG11 is not a linkage group but contains markers MA118 and RB12 which were not assignable to any of the linkage groups above

4.3.3. *QTL identification for low P tolerance*

Significant QTL for low P tolerance were identified in LG 3A. The LOD traces, indicative of QTL, for each phenotyping data set analyzed are shown in Fig. 26. Two QTL, *QAB.tam-3Chr.1* (14.47-39 cM) and *QAB.tam-3Chr.2* (68.58-119.48 cM), were identified from qualitative scores for low P tolerance (Fig. 27A). One QTL, *QLP.tam-3Chr* (72.58-120 cM), was identified from shoot dry biomasses in the low P treatment (Fig. 27B). One QTL, *QPSI.tam-3Chr* (43-118.48 cM), was identified from PSI values (Fig. 27C). From Mapchart 2.2 (Wageningen UR, Wageningen, Netherlands), a map of the location of each QTL is shown in Fig. 28 (Voorrips 2002). There is overlap in the location of *QAB.tam-3Chr.2*, *QLP.tam-3Chr*, and *QPSI.tam-3Chr* suggesting these QTL may represent the same gene(s) of interest for low P tolerance. Also, there is an increase in LOD score that corresponds to QTL *QAB.tam-3Chr.1* from low P treatment shoot biomass and PSI data (Fig. 27), but the LOD score increase was not significant enough to indicate a QTL. More markers located in LG3A need to be run on additional RIL lines to further fine-map the QTL identified. A summary of the QTL identified and their associated markers, Kruskal-Wallis significance values, LOD scores, R^2 values, and additive effects are given in Table 19.

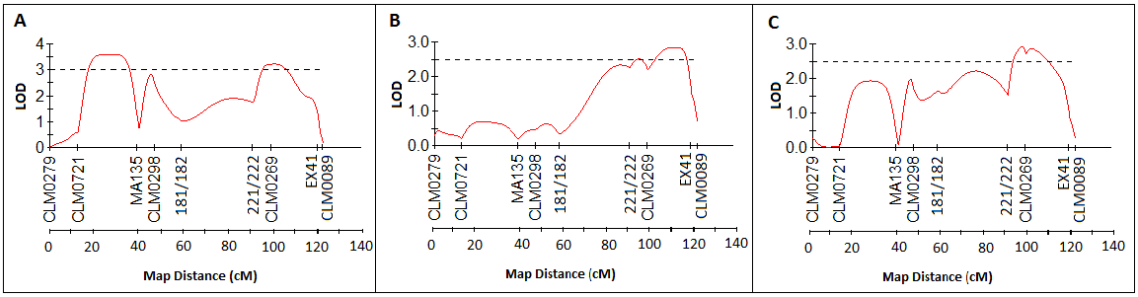

Fig. 26 LOD traces for QTL identified for low P tolerance. QTL identified were from qualitative positive/negative scores for low P tolerance (A), mean shoot biomass in a low P treatment (B), and PSI (C). Markers and map distances are also indicated. LOD thresholds of 3 (A) and 2.5 (B and C) are indicated by broken lines

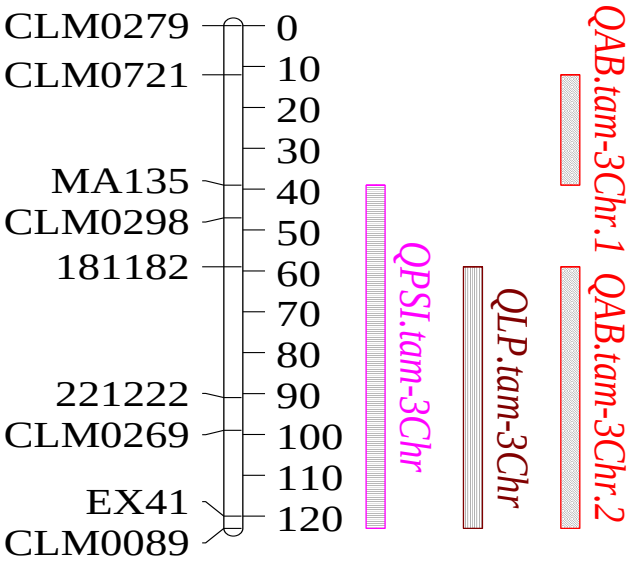

Fig. 27 Location of QTL identified for low P tolerance in LG3A for cowpea

Table 19 Map position, relevant markers, Kruskal-Wallis significance, LOD, LOD Threshold, R^2, and additive effects for each low P tolerance QTL identified

QTL	Data type	LG	Position (cM)	Relevant Markers	Kruskal-Wallis Sig. Level	LOD	LOD Threshold	R^2	Additive Effect
QAB.tam-3Chr.1	Positive or negative qualitative scores for low P tolerance	3	14.47-39	CLM0721, MA135	.	1.27-3.60	3	7.7-15.7	-0.01--0.09
QAB.tam-3Chr.2	Positive or negative qualitative scores for low P tolerance	3	68.58-119.48	181/182, 221/222, CLM0269, EX41, CLM0089	0.01-0.0001	1.4-3.24	3	6.4-14.2	-0.02--0.25
QLP.tam-3Chr	Mean shoot biomass in a low P treatment	3	72.58-120	181/182, 221/222, CLM0269, EX41, CLM0089	0.01-0.0001	1.45-2.84	2.5	6.7-12.6	-0.03--0.12
QPSI.tam-3Chr	P susceptibility index (PSI)	3	43-118.48	MA135, CLM0298, 181/182, 221/222, CLM0269, EX41, CLM0089	0.01-0.0001	1.37-2.92	2.5	7-14.5	0.04-0.31

Table 19 lists relevant markers, border and interior, for the QTL identified for low P tolerance. However, markers that can be used for direct selection of low P tolerance are of highest value. Kruskal-Wallis significance results from MIM in ICIM for all phenotyping results indicated two markers, CLM0269 and 221/222, can be used for selection of cowpea varieties for low P tolerance because of a high correlation between marker and phenotyping results. In addition, another marker CLM0298, according to just PSI results, can be used for selection for low P tolerance because of a high correlation between marker and phenotyping results. Phenotyping results organized by each marker's inheritance from either IT98K-476-8, positive for low P tolerance, or Aloka, negative for low P tolerance, across the F_6 RIL population are shown in Fig. 28. Results in Fig. 28 confirm these markers as valuable for selection for low P tolerance in cowpea.

Markers CLM0298 and CLM0269 have been previously identified as linked, 11 cM apart, according to a linkage map of Xu *et al*. (2011). It is suspected that LG3 published in Xu *et al*. (2011) correlates with this study's LG3A and LG3B, and in this LG is the gene(s) responsible for low P tolerance. Also, marker 221/222 was identified as a part of LG2 of Andergie *et al*. (2011), but the other markers tested from LG2 of Andergie *et al*. (2011) were not positive indicators of low P tolerance.

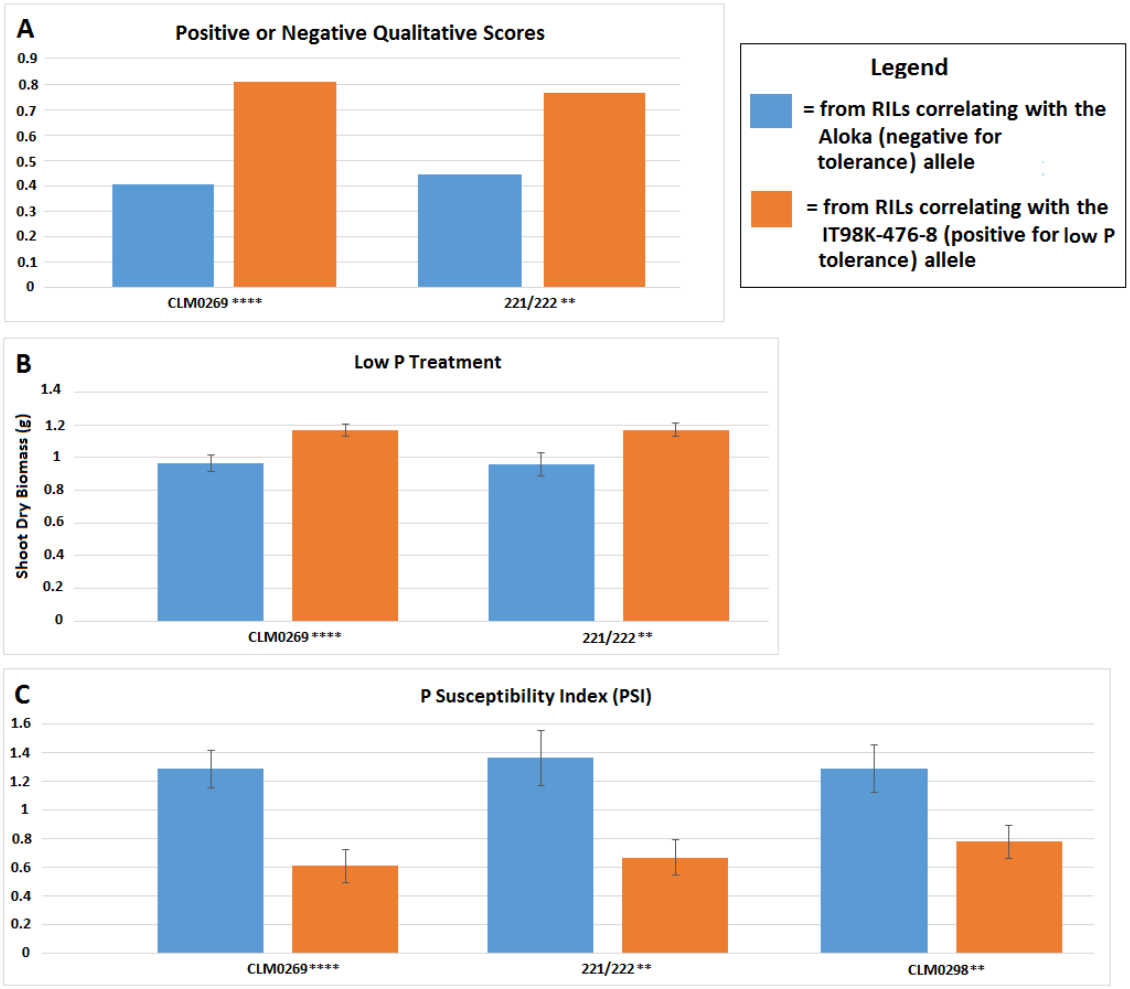

Fig. 28 Graphical summary of the F_6 RIL population phenotyping results organized according to markers that can be used to select for low P tolerance. ** is Kruskal-Wallis sig. of 0.01, and **** is Kruskal-Wallis sig. of 0.0001. Error bars represent standard error

4.4. Discussion

QTL mapping for low P tolerance with an F_6 RIL population of 120 individuals and 75 SSR markers has successfully identified QTL in LG3A (Fig. 28), in which gene(s) responsible for low P tolerance are located. This linkage group is suspected to

correlate with LG3 of Xu *et al*. (2011). Three of the four QTL identified overlap and may represent the same genes of interest. Further fine-mapping with additional markers and RILs will strengthen QTL results.

From these QTL, two markers, CLM 0269 and 221/222, were identified as correlating to low P tolerance across all phenotyping data. A third marker CLM0298 was identified as correlating to low P tolerance from PSI data. These markers can be used for positive selection of cowpea varieties for low P tolerance. These results pave the way for marker assisted selection (MAS), which is valuable for selecting desired traits in crop varieties while minimizing phenotyping costs and time.

Further fine-mapping of the low P tolerance trait via further analysis with additional markers and RIL lines or populations is suggested to further support and enhance positive marker results for low P tolerance. Of particular interest will be additional markers associated with LG3A in Fig. 25 and LG3 in Xu *et al*. (2011).

5. CONCLUSIONS

Cowpea varieties were successfully phenotyped for low P tolerance by growing them in silica sand-filled pots watered with nutrient solutions of no P, low P, or normal P treatments. Low P tolerance was identified in four cowpea varieties: Big John, IT97K-1069-6, IT98K-476-8, and TX2028-1-3-1. In addition, partial low P tolerance through high seed P content was identified in Big John, CB-46, and Golden Eye Cream. Cotyledon removal to study the effect of high seed P on cowpea varieties did not show any significant differences among varieties, suggesting that the advantage of high seed P is already in effect before seedlings emerge from soil. Increases in root growth for several varieties when exposed to low P treatments were identified, but this increased root growth did not necessarily confer low P tolerance, suggesting other physiological mechanisms to be acting in addition to root growth to confer low P tolerance. Internal shoot P content results showed that under the low P treatment, all varieties had suboptimal shoot P, and under the normal P treatment, all varieties had abundant shoot P. Though internal shoot P contents under the low P treatment were suboptimal for all varieties, of these varieties IT98K-476-8 followed by IT97K-1069-6 had the highest internal shoot P content, further suggesting these varieties as having low P tolerance.

Significant additive effects and high narrow-sense heritability were identified for low P tolerance in cowpea according to results from phenotyping the F_1, BC_1, and F_2 seed from high by low crosses of cowpea for low P tolerance via the silica sand and nutrient solution phenotyping method used in Section 2 on cowpea varieties. The very

high narrow sense heritability indicated simple inheritance for this trait. This was further confirmed by estimating the number of genes involved using the mean differences between the tolerant and susceptible parents and their F2 genetic variances. The results indicated one major gene controlling this trait in both crosses. Thus, the low P tolerance can be easily transferred and fixed into improved cowpea lines by breeding efforts.

Linkage group and QTL mapping for low P tolerance identified QTL in LG3A for low P tolerance. Three markers – CLM0269, 221/222, and CLM0298 – were identified as candidate markers for MAS within these QTL. Further fine-mapping with additional markers and additional RIL phenotyping is desired.

Together these results contribute knowledge to breeding cowpea for the P-deficient soils of Sub-Saharan West Africa via identifying tolerant varieties and elucidating a few of the potential physiological mechanisms responsible for tolerance. These results suggest that tolerant cowpea lines via low P tolerance can be readily developed since low P tolerance is a heritable additive trait. In addition, QTL for low P tolerance in cowpea were identified that pave the way for future fine-mapping and potential MAS for low P tolerance.

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